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(54) Title: TISSUE-DERIVED TUMOR GROWTH INHIBITORS, METHODS FOR PREPARATION AND USES THERE-OF (57) Abstract <p>The present invention provides methods for producing and purifying proteins comprising TGF-β3 precursor, pro region of the TGF-β3 precursor and mature TGF-β3. Moreover, this invention also provides methods for producing and purifying novel homodimeric proteins with enhanced production and heightened therapeutic utility which comprises mutations, signal peptide deletions, and protease cleavage site substitutions.</p>		

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1

**TISSUE-DERIVED TUMOR GROWTH INHIBITORS, METHODS FOR
PREPARATION AND USES THEREOF**

This application is a continuation-in-part of U.S. Serial No. 353,410, filed May 17, 1989, which is a continuation-in-part of U.S. Serial No. 183,224, filed April 20, 1988, which is a continuation in part of U.S. Serial No. 111,022, filed October 20, 1987, which is a continuation-in-part of U.S. Serial No. 922,121, filed October 20, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 847,931, filed April 7, 1986, now abandoned, which was a continuation-in-part of U.S. Serial No. 725,003, filed April 19, 1985, now abandoned, the contents of each are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Transforming growth factor β (TGF- β) is part of a family of multifunctional proteins which appear to modulate, alone or in combination with other molecules, cell proliferation and differentiation. Reportedly, TGF- β , which comprises both a mature, a pro and a precursor form, includes several isoforms (i.e. TGF- β 1, - β 2, - β 3, - β 4, and - β 5) coded for by different

g n s. The TGF- β 2 and TGF- β 3 cDNA have been found from several mammalian sources (14). TGF- β 4 cDNA has only been isolated from chicken cells (17). Mature TGF- β 1, - β 2, - β 3 cDNAs share an amino acid sequence identity of about 75-80% whereas the TGF- β 1, - β 2, - β 3 precursor exhibit only 25-35% identity. Surprisingly, despite the high degree of homology among the TGF- β 's, it appears that these proteins have distinct differences in potencies (14).

Mature TGF- β has been isolated from various species. Murine, bovine, human, and porcine TGF- β have been isolated and show very little difference in amino acid composition (5, 8, 11, 14, 24).

The cDNA sequence of mature TGF- β , its expression in both normal and transformed cells, and methods for producing biologically active mature TGF- β in eucaryotic cells have been described (2, 37, 8, 11, 38).

R. Derynck et al. (38) have described a method comprising (a) constructing a vector which includes nucleic acid encoding TGF- β 3, (b) transforming a heterologous host eucaryotic cell with the vector, (c) culturing the transformed cell, and (d) recovering TGF- β 3 from the culture medium.

Summary of the Invention

The present invention provides methods for producing and purifying proteins comprising TGF- β 3 precursor, pro region of the TGF- β 3 precursor and mature TGF- β 3. Moreover, this 5 invention also provides methods for producing and purifying novel homodimeric proteins with enhanced production and heightened therapeutic utility which comprises mutations, signal peptide deletions, and protease cleavage site substitutions.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence encoding TGF- β 3 and its deduced amino acid sequence. Putative glycosylation sites and polyadenylation signal are underlined. The start of the mature TGF- β 3 is marked by an asterisk at alanine at nucleotide positions 1163-1165.

Figure 2 shows a Southern blot analysis of human tumor DNAs hybridized with a PvuII-PvuII TGF- β 1 cDNA probe.

Figure 3 shows a Northern blot analysis of A673, A549, and A498 cell lines using an EcoRI-BglII 1.7kb cDNA fragment of the gene encoding TGF- β 3 as a probe.

Figure 4 shows a Northern blot analysis of A673, A549, and A498 cell lines using a PvuII-TaqI probe from genomic sequences of TGF- β 3.

Figure 5 shows a Northern blot analysis of A673, A549, and A498 cell line using a PstI-BalI TGF- β 1 probe.

Figure 6 shows a Northern blot analysis of A673, A549, and A498 cell lines using TGF- β 1 cDNA containing the complete coding sequence of TGF- β 1 precursor as a probe.

Figure 7 shows a Northern blot analysis of mRNA from umbilical cord and A673 cell lines using an EcoRI-BglII cDNA fragment of the gene encoding TGF- β 3 as a probe.

Figure 8 shows the production of trpE::TGF- β 3 fusion protein of three lysates by SDS polyacrylamide gel electrophoresis. (A) corresponds to TGF- β 3.

Figure 9 shows whole cell bacterial lysates containing

trpE::TGF- β 1 fusion proteins (lanes 1 and 4), trpE::(A) fusion proteins (lanes 2 and 5), and the TGF- β 1 protein (purchased from R&D Systems) (lanes 3 and 6) were separated on a 12.5% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter (1 μ m pore size) and incubated with 100 μ g of affinity purified anti-peptide antibody either in the absence (lanes 1, 2 and 3) or presence of a 300 fold molar excess of the antigenic peptide (lanes 4, 5, and 6). The antibodies were detected using alkaline phosphatase conjugated to goat anti-rabbit antibody (Promega) according to the manufacturers instruction.

Figure 10 shows a schematic diagram of mRNA encoding TGF- β 3 with the coding sequence boxed. The relative extension of the cDNA inserts obtained from placenta (1.7 kb), umbilical cord (1.9 kb) and A673 (1.7 kb) libraries is indicated. The dashed part of the box represents the C-terminal region showing high homology to TGF- β s. The 5' EcoRI-Bgl II restriction fragment of the placenta cDNA is indicated by a bar.

Figure 11 A/B shows a comparison of the nucleotide sequence and predicted amino acid sequence of the gene encoding TGF- β 3 with TGF- β 1 and TGF- β 2. Identical amino acids are boxed. The mature TGF- β 3 amino acid sequences start at position 315. (A) corresponds to TGF- β 3.

Figure 12 is a schematic representation of the construction of the pCMV-TGF- β 3 expression plasmid from pORFX and pBlue-TGF- β 3 plasmids.

Figure 13 shows the level of TGF- β 3 mRNA expression, determined by Northern hybridization using a TGF- β 3 specific probe, of parental CHO cells (lane 1), CHO cells transfected with TGF- β 3 cDNA (CHO 6.35) (lane 2) and CHO 6.35 amplified with 20nM MTX (CHO 6.35/20nM (lane 3).

Figur 14

(A) shows the dose response of mink cell growth inhibition using purified TGF- β 1. Cell growth was quantitated by the metabolism of MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetraazolum bromide; Thiazolyl blue) (148).

(B) shows the dose response of mink cell growth inhibition using acid activation serum free supernatants CHO 6.35/20nM transfectant and CHO 6.35 transfectant. Cell growth was quantitated by the metabolism of MTT.

10 **Figure 15** shows the relative location of the various TGF- β 3 peptides used as antigens.

Figure 16 shows the immunoprecipitation of native recombinant TGF- β 3 protein by β 3V antibody.

Figure 17

15 (A) shows the immunoblot of TGF- β 3 from conditioned media of CHO 6.35/20nM transfectant using β 3III and β 3V antibodies for detection from gels under reducing conditions.

(B) shows the immunoblot of TGF- β 3 from conditioned media of CHO 6.35/20nM transfectant using β 3III and β 3V antibodies for
20 detection from gels under non-reducing conditions.

Figure 18 shows a Western blot of cell extract (18A) and conditioned media (18B) of the CHO 6.35/20nM transfectant using β 3V antibody for detection.

Figure 19 A,B,C,D shows the staining to paraffin sections of
25 human umbilical cord by β 3V antibody and control antibody. A and C show fibroblast and epithelial staining and smooth muscle fiber staining, respectively, by β 3V antibody. B and D show no staining by control rabbit polyclonal antibody.

Figure 20 is a silver stained gel of purified TGF- β 3 and TGF- β 1.

Figure 21 A, B, C shows specific antibody neutralization of TGF- β 3 inhibition of mink cell growth by 83V.

5 Figure 22 shows the location of the various protease sites genetically engineered into the TGF- β 3 precursor.

Detailed Description of the Invention

In accordance with the invention, mature TGF- β 3 is defined as a recombinant homodimeric protein which comprises two polypeptides each of which consists essentially of 112 amino acids and has a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498.

Moreover, as used herein TGF- β 3 precursor is a recombinant homodimeric protein which comprises two polypeptides, each polypeptide encoded by a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a serine encoded by nucleotides 1496-1498.

Further, as used herein the pro region of the TGF- β 3 precursor is a recombinant protein which comprises the TGF- β 3 precursor without the mature TGF- β . In particular, the pro region of the TGF- β 3 precursor is a protein encoded by a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an methionine encoded by nucleotides 263-265 and ending with a arginine encoded by nucleotides 1160-1162.

Also, as used herein, reference to TGF- β means either mature TGF- β (e.g. TGF- β 1, - β 2, - β 3), TGF- β precursor (e.g. TGF- β 1 precursor, TGF- β 2 precursor, TGF- β 3 precursor), or the pro region of the TGF- β (e.g. TGF- β 1, - β 2, - β 3) precursor.

The present invention provides a method of recovering purified, non-denatured mature TGF- β 3 from a mixture of mammalian cell-derived polypeptides. The method comprises contacting the mixture with an antibody which specifically

binds to mature TGF- β 3 but exhibits substantially no cross reactivity with mature TGF- β 1 and mature TGF- β 2.

In one example, the mixture of mammalian cell-derived polypeptides is a mixture of non-human mammalian polypeptides from non-human cells in which TGF- β 3 has been expressed.

Additionally, in another example of the subject invention, the antibody may be directed to an epitope defined by the amino acid sequence YLRSDTTHSTVLGLYNTLNPEASASY. By way of example, the previously described antibody may be immobilized on a solid support under conditions such that TGF- β 3 is isolated and purified.

Additionally, this invention provides a method for producing substantially purified TGF- β 3 precursor having an anchorage membrane sequence which comprises: (a) preparing DNA encoding a TGF- β 3 precursor having the membrane anchorage sequence; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF- β 3 precursor is expressed and subsequent translocation of the expressed TGF- β 3 precursor having the membrane anchorage sequence; (d) culturing the host cell in medium; (e) separating the host cell from the medium; (f) disrupting the cell such that a lysate containing the TGF- β 3 precursor having the membrane anchorage sequence is produced; and (g) purifying the TGF- β 3 precursor having a membrane anchorage sequence from the lysate under conditions such that the substantially purified TGF- β 3 precursor is produced.

The present invention also provides a method for producing substantially purified TGF- β 3 precursor having an anchorage membrane sequence. The method comprises: (a) preparing DNA

encoding the TGF- β 3 precursor having a membrane anchorage sequence; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF- β 3 precursor is expressed and subsequent occlusion of the expressed TGF- β 3 precursor having a membrane anchorage sequence in occlusion bodies; (d) culturing the host cell in culture medium; (e) separating the occlusion bodies from the host cells and the culture medium; (f) disrupting the occlusion bodies to produce a solution containing the TGF- β 3 precursor having a membrane anchorage sequence; and (g) purifying the resulting TGF- β 3 precursor having a membrane anchorage sequence from the lysate under conditions such that the substantially purified TGF- β 3 precursor is produced. The above-described method further comprises: (a) treating the purified TGF- β 3 precursor as recovered with an activating agent to separate a mature TGF- β 3 from the precursor; and (b) recovering the separated mature TGF- β 3 of step (a).

In accordance with the present invention, the anchorage linkage sequence may be a phosphatidyl inositol linkage. Alternatively, the anchorage linkage sequence is a hydrophobic transmembrane peptide sequence.

Also, the present invention provides a method for producing a substantially purified mutant TGF- β 3 precursor. The method comprises (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence

substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell of step (b) is transformed with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF- β 3 precursor is expressed; (d) culturing the host cell in medium under conditions such that the mutant TGF- β 3 precursor so expressed is secreted into the medium; (e) separating the host cell from the culture medium containing the mutant TGF- β 3 precursor so secreted; and (f) purifying the mutant TGF- β 3 precursor such that a substantially purified mutant TGF- β 3 precursor is produced. The above-described method further comprises: (a) treating the purified mutant TGF- β 3 precursor so recovered with an activating agent to separate a mature TGF- β 3 from the precursor; and (b) recovering the separated mature TGF- β 3 of step (a).

Moreover, this invention also provides a method for producing a substantially purified mutant TGF- β 3 precursor. The method comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498; inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host

cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF- β 3 is expressed; (d) separating the host cell containing the expressed, mutant TGF- β 3 precursor from the culture medium; (e) disrupting the host cell such that a lysate containing the mutant TGF- β 3 precursor is produced and (f) purifying the mutant TGF- β 3 precursor such that a substantially purified mutant TGF- β 3 precursor is produced. Additionally, the above-described method further comprises (a) treating the purified mutant TGF- β 3 precursor so recovered with an activating agent to separate a mature TGF- β 3 from the precursor and (b) recovering the separated mature TGF- β 3 of step (a).

Additionally, this invention provides a method for producing a substantially purified mutant TGF- β 3 precursor. The method comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the

vector in order to induce expression of the DNA of step (b) such that a mutant TGF- β 3 precursor is expressed; (d) culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 precursor is secreted into the medium; (e) separating the cell from the culture medium containing the mutant TGF- β 3 precursor so secreted; and (f) purifying the mutant TGF- β 3 precursor such that a substantially purified mutant TGF- β 3 precursor is produced. The method further comprises: (a) treating the purified mutant TGF- β 3 precursor so recovered with cyanogen bromide to separate a mature TGF- β 3 from the precursor; and (b) recovering the separated mature TGF- β 3 of step (a).

Further, the invention also provides a method for producing a substantially purified mutant TGF- β 3 precursor which comprises: (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b)

such that a mutant TGF- β 3 precursor is expressed; (d) separating the host cell containing the expressed, mutant TGF- β 3 precursor from the culture medium; (e) disrupting the host cell such that a lysate containing a mutant TGF- β 3 precursor is produced; and (f) purifying the mutant TGF- β 3 precursor such that a substantially purified mutant TGF- β 3 precursor is produced. The method further comprises: (a) treating the purified mutant TGF- β 3 precursor so recovered with cyanogen bromide to separate a mature TGF- β 3 from the precursor; and (b) recovering the separated mature TGF- β 3 of step (a).

Further, in accordance with the practice of the above-described methods, X in step (a) may be selected from a group of tri-nucleotides consisting of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT, TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT, CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, ATA, ACT, ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG, GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA, GAG, GGT, GGC, GGA, and GGG.

In one embodiment of the subject invention, purification is effected by affinity chromatography. One example of affinity chromatography is antibody column chromatography. Another example of affinity chromatography is lectin column chromatography. Lectin column chromatography allows isolation of the glycosylated precursor form of TGF- β 3.

Also, in one example of the invention, in step (a) of the above-described method, the protease cleavage sequence may be a collagenase recognition sequence. Alternatively, the protease cleavage sequence may be a Factor Xa recognition sequence. The protease cleavage sequence allows for the production of a membrane anchored TGF- β 3 precursor.

This invention also provides a method for producing mutant

TGF- β 3. The method comprises (a) preparing a DNA comprising a sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with leucine encoded by nucleotides 332-334 and ending with serine encoded by 5 nucleotides 1496-1498; (b) inserting the DNA of step (a) into an expression vector operably linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF- β 3 is expressed; (d) culturing 10 the host cell in medium; (e) separating the host cells containing the mutant TGF- β 3 so expressed from the medium; (f) disrupting the cells to produce a lysate containing the mutant TGF- β 3; and (g) purifying the mutant TGF- β 3. The above-described method further comprises (a) treating the purified 15 mutant TGF- β 3 so recovered with an activating agent to separate a mature TGF- β 3 from the mutant TGF- β 3; and (b) recovering the separated mature TGF- β 3 of step (a).

This invention additionally provides a process which comprises: (a) contacting a TGF- β 3 precursor with a 20 precipitating agent thereby concentrating the TGF- β 3 precursor in a precipitate; (b) extracting the pellet of step (a) with an acidified organic solution under such conditions that mature TGF- β 3 is separated from the pellet; and (c) recovering the mature TGF- β 3 so separated in step (b).

25 In accordance with the subject invention, the acidified organic solution in step (b) may be an acidified acetonitrile. Additionally, the organic solution may comprise 50% acetonitrile and 1.0M acetic acid. Also in accordance with the subject invention, the precipitating agent in step (a) may 30 be ammonium sulfate.

This invention also provides a method for producing and identifying a mutant, mature TGF- β 3 exhibiting reduced binding

affinity to serum binding proteins. The method comprises (a) preparing a DNA encoding the TGF- β 3; (b) performing mutagenesis (for example random mutagenesis) on the DNA of step (a) thereby obtaining a mutant DNA; (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell; (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF- β 3 is expressed; (e) culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 is secreted into the medium; (f) separating the host cell from the culture medium containing the mutant TGF- β 3 so expressed; (g) purifying the mutant TGF- β 3; (h) activating the mutant TGF- β 3 so expressed under conditions such that a mutant, mature TGF- β 3 is separated from the mutant TGF- β 3; and (i) assaying the culture medium for the mutant mature TGF- β 3 thereby identifying a mutant mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins.

Additionally, this invention further provides a method for producing and identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins. The method comprises: (a) preparing a DNA encoding the TGF- β 3; (b) performing mutagenesis (for example random mutagenesis) on the DNA of step (a) thereby obtaining a mutant DNA; (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell; (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF- β 3 is expressed; (e) culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 is produced in the host cell; (f) separating the host cell containing the mutant TGF- β 3 so expressed from the culture medium; (g) disrupting the cells to produce a lysate containing the mutant TGF- β 3; (h) purifying the mutant TGF- β 3;

(i) activating the mutant TGF- β 3 so expressed under conditions such that a mature TGF- β 3 is separated from the mutant TGF- β 3; and (j) assaying the culture medium for the mutant mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins thereby identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins.

In one example of the above-described method, the serum binding protein is α 2-macroglobulin. Alternatively, in another example, the serum binding protein is type III TGF- β receptor, e.g. betaglycan. Further alternatively, the serum binding protein may be a pro region of the TGF- β precursor.

This invention further provides a method for producing a substantially purified pro region of the TGF- β 3 precursor. The method comprises: (a) preparing DNA encoding the pro region of the TGF- β 3 precursor; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF- β 3 precursor is expressed; (d) culturing the host cell in medium; (e) separating host cells from the medium; (f) disrupting the host cells to produce a lysate containing the pro region of the TGF- β 3 precursor; and (g) purifying the pro region of the TGF- β 3 precursor from the lysate such that the substantially purified pro region of the TGF- β 3 precursor is produced.

Additionally, this invention provides a method for producing a substantially purified pro region of the TGF- β 3 precursor which comprises: (a) preparing DNA encoding the pro region of the TGF- β 3 precursor; (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell; (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions

such that a pro region of the TGF- β 3 precursor is expressed;
(d) culturing the host cell in medium under conditions such
that the expressed pro region of the TGF- β 3 precursor is
secreted into the medium; (e) separating host cells from the
5 medium containing the pro region of the TGF- β 3 precursor so
secreted; and
(f) purifying the pro region of the TGF- β 3 precursor such that
the substantially purified pro region of the TGF- β 3 precursor
is produced.

10 Further, in each of the above-described methods, the host cell
may be either a eucaryotic cell or a procaryotic cell.

This invention is illustrated in the Experimental Details
section which follows. This section is set forth to aid in an
understanding of the invention but is not intended to, and
15 should not be construed to, limit in any way the invention as
set forth in the claims which follow.

EXPERIMENTAL DETAILS**Abbreviations and Technical Terms:**

- AL (acute leukemia)
ANLL (adult non-lymphocytic leukemia)
5 APRT (adenosylphosphoribosyl transferase)
BFU-E (burst forming unit-erythroid)
BSA (bovine serum albumin)
CL (chronic leukemia)
CLL (chronic lymphocytic leukemia)
10 CML (chronic myelogenous leukemia)
CNBr (cyanogen bromide)
CFU (colony forming unit)
CFU-E (colony forming unit-erythroid)
CFU-GEMM (colony forming unit-granulocyte, erythroid,
15 macrophage, monocyte)
CFU-GM (colony forming unit-granulocyte/macrophage)
CFU-meg (colony forming unit-megakaryocyte)
CHO (chinese hamster ovary)
CMV (cytomegalovirus)
20 CSF (colony stimulating factor)
DHFR (dihydrofolate reductase)
DMEM (Dulbecco's modified Eagle's medium)
DMF (dimethyl formamide)
DMSO (dimethyl sulfoxide)
25 DNA (deoxyribonucleic acid)
EPO (erythropoietin)
FCS (fetal calf serum)
G-CSF (granulocyte-colony stimulating factor)
GM-CSF (granulocyte/macrophage-colony stimulating factor)
30 kb (kilobase pairs)
kDa (kil Daltons)
HPLC (high pressure liquid chromatography)

- IL-3 (interleukin-3)
- IL-4 (interleukin-4)
- MEM (modified Eagle's medium)
- mRNA (messenger ribonucleic acid)
- 5 RNA (ribonucleic acid)
- TGF- β (transforming growth factor-beta)
- TIF (tumor inhibitory factor)
- WBC (white blood cell)

EXAMPLE 1: CLONING THE GENE ENCODING TGF- β 3

- 10 To identify sequences with homology to TGF- β 1 a Pvu II-Pvu II probe, containing most of the mature form of the TGF- β 1 cDNA sequence, was 32 P labelled and used to screen a Southern blot (34) of total human DNAs digested with Eco RI, Hind III or Sst I using standard methods. In each digest, two bands were
- 15 present at a low stringency wash (2.5 x SSC, 65°C) (Figure 2). When the wash stringency was increased (0.01 x SSC, 65°C) only one hybridizing band remained in each digest (Figure 2). The strongly hybridizing band is TGF- β 1, and the weakly hybridizing band is a related gene which also encodes TGF β 3.
- 20 The nucleotide sequence encoding TGF- β 3 and its amino acid sequence are shown in (Figure 1).

To isolate the gene encoding TGF- β 3 with homology to TGF- β 1, the TGF- β 1 clone was used to screen a human phage library constructed from the DNA of a chronic myelocytic leukemia cell

25 line (K562). Two genomic loci, which correspond to TGF- β 1 and the related gene encoding TGF- β 3 (Figure 1), were cloned. Construction of the K562 library, screening and isolation of recombinant clones was carried out essentially according to the procedures of Grosveld, et al. (15).

- 30 The phage DNA clone containing the sequence encoding TGF β 3, was cut with Sau 3A and the restriction fragments cloned into

M13. The recombinant plaques were screened with the SmaI-PvuII probe of TGF- β 1. Six hybridizing genomic clones were sequenced by the method of Sanger, et al. (33) and a region of approximately 130 bp was found to be homologous to TGF- β 1 cDNA. When the amino acid sequence of TGF β 1 and TGF β 3 cloned in these experiments were compared they were found to be 82% homologous.

To obtain a repeat free probe of TGF- β 3 various restriction fragments from BamHI-BamHI subclone of this gene were hybridized to TGF- β 1 cDNA, as well as to total human DNA. A BamHI-TaqI fragment of the gene, i.e. TGF β 3, was found to cross hybridize with the TGF- β 1 cDNA but did not hybridize to repetitive sequence elements in human DNA.

The BamHI-TaqI unique probe of the sequence encoding TGF- β 3 was used to screen the lambda-gt11 human placenta cDNA library (Clontech). Two strongly hybridizing clones, as well as four weakly hybridizing clones, were isolated. By DNA sequence analysis the weakly hybridizing clones were shown to correspond to TGF- β 1. One strongly hybridizing clone was isolated and a 1.7kb EcoRI insert was subcloned into pUC 8.

Restriction fragments for this clone were subcloned into M13 and sequenced by the method of Sanger, et al. The deduced amino acid sequence of this gene exhibits extensive homology with a family of genes (24) including TGF- β 1, TGF- β 2, glioblastoma T-cell suppressor factor (G-TsF), inhibin/activin, Mullerian Inhibiting Substance (MIS) and decapentaplegic transcript complex of Drosophila with the six C-terminal cysteine residues being conserved throughout.

A 17 kb genomic DNA fragment containing the sequence of the gene, i.e. TGF- β 3, has been cloned. Hybridizing 5' and 3' portions of the 1.7 kb cDNA clone which encodes TGF- β 3 with

the genomic locus of TGF- β 3 revealed that the 1.7 kb cDNA sequence is completely contained in the genomic clone. Taking into account that the full length message of TGF- β 3 is 3.5 kb, additional 5' and 3' flanking sequences need to be isolated to obtain the complete gene. This is done by screening genomic phage and cosmid libraries with probes unique to the gene encoding TGF- β 3.

In TGF- β 1 the sequence R-R represents the proteolytic cleavage site which generates the mature protein. In TGF- β 3 the sequence R-K-K-R represents the corresponding cleavage site.

In the region N-terminal to the cleavage site, TGF- β 1 and TGF- β 3 exhibit only 35% homology. However, both proteins contain the sequence R-G-D in the N-terminal region which is recognized by the fibronectin receptor.

In order to determine which cell types express TGF- β 3, Northern hybridization was carried out using a 5' terminal EcoRI-BglII probe (Figure 3). The result of Northern hybridization revealed a mRNA of approximately 3.5 kb in A673 (a rhabdomyosarcoma), A498 (a kidney carcinoma) and a faintly hybridizing signal in A549 (a lung adenocarcinoma).

A genomic probe from the 3' region of TGF β 3 (corresponding to sequences downstream of the presumed site of proteolytic cleavage) was then used to screen the same Northern blot. Three strong hybridization signals were observed in both A673 and A498, corresponding to TGF- β 1 (2.5 kb), TGF- β 3 (3.5 kb) and another related gene (4.2 kb) (Figure 4). These results are consistent with the notion that this probe cross reacts with sequences homologous to TGF- β 3.

Northern blot analysis of A673, A549 and A498 cell lines using a PstI-BalI TGF- β 1 probe was then performed. The PstI-BalI

TGF- β 1 probe strongly hybridized to a 2.5 kb mRNA band in all three cell lines. Several weakly hybridizing bands are also observed at 4.2 kb and 3.5 kb (Figure 5). This probe is highly specific for TGF- β 1 since it contains sequences 5 corresponding to those residues N-terminal to the proteolytic cleavage site, a region where TGF- β 1 exhibits little homology to other members of this gene family.

A Northern blot of A673, A549 and A498 cell lines were then screened using TGF- β 1 cDNA containing the complete coding 10 sequence of the TGF- β 1 precursor. This probe cross hybridized with homologous sequences to TGF- β 1. Specifically, there was strong hybridization to a 2.5 kb mRNA band corresponding to TGF- β 1 (Figure 6).

Northern blot analysis of mRNA from human umbilical cord and 15 the A673 cell line was also screened using an EcoRI - BglII cDNA fragment of TGF- β 3 as a probe (Figure 7). Figure 7 also illustrates the result of a Northern blot using an actin probe as a control to normalize mRNA levels in each lane. When normalized to actin mRNA levels, the umbilical cord expresses 20 the highest level of mRNA of the gene encoding TGF- β 3 in comparison to other mRNA sources so far examined.

Southern blot analysis was performed on a variety of different tumor DNAs digested with EcoRI and hybridized with a SmaI-AvaI cDNA fragment of TGF- β 3 as a probe. Hybridization was 25 effected at both low (2.5 X SSC, 65°C) and high (0.3 X SSC, 65°C) stringency washes. Southern blot analysis indicated the possible presence of other loci related to TGF- β 3. The probe hybridized with two bands (3 kb and 12 kb) which were observed only if washed with low stringency.

Producti n of Antibodies with Specificity for TGF- β 3

Chimeric bacterial proteins, i.e. fusion proteins, have been constructed which contain the C-terminal 150 amino acids of TGF- β 3 fused to a small region of the trpE gene. The fusion protein was recognized by an antibody produced against a peptide derived from amino acid numbers 9 to 28 of the mature form of TGF- β 3. The antibody recognized the trpE::TGF- β 3 fusion protein and the peptide specifically competes with TGF- β 3 for the binding of the antibody.

10 DNA sequences that code for TGF- β 3 were cloned into a pKS vector. This vector is a pATH II derivative that contains the inducible trp promoter and a multiple cloning site. The resulting constructs produce a chimeric protein consisting of the first 22 amino acids of the trpE gene, the C-terminal 150 amino acids of TGF- β 3.

Transformants containing these clones were screened primarily by restriction endonuclease analysis and ultimately for production of the chimeric protein by SDS polyacrylamide gel electrophoresis. The protein products of 3 clones, p116, 20 p134, and p135, are shown in Figure 8. These cells were grown in defined media until they reached early log phase and then incubated for 3 hours either in the presence or absence of the trpE inducer indoleacrylic acid (IAA). The cells were then collected, lysed and their proteins electrophoresed on a 12.5% 25 SDS polyacrylamide gel. Figure 8 is a photograph of one such gel that had been stained with Coomassie blue. As can be seen, lysates p116 and p135 produce a protein of about 19,000 Dalton molecular weight whose relative abundance increases in the presence of IAA. In contrast, p134 does not produce this 30 protein species. Both p116 and p135 contain plasmids that, by restriction analysis, have the sequences of TGF- β 3 cloned in

the orientation that should produce a 19,500 Dalton molecular weight fusion protein. The p134 plasmid was found to have the sequences of TGF- β 3 in the opposite orientation.

The trpE::TGF- β 3 fusion protein was used to test the specificity of an antibody that used a peptide homologous to part of TGF- β 3 as an antigen. A polypeptide was synthesized corresponding to residues 9 through 28 of mature TGF- β 3 except that residue 9 in the sequence, arginine, was replaced by serine. The peptide was purified by reverse phase HPLC and coupled to keyhole limpet hemocyanin for use as an immunogen in rabbits.

Thirty-three days following the first injection (500 μ g), the antisera were screened by standard ELISA using 100ng of peptide per well. One rabbit demonstrated a signal of 1.0 OD units at a 1:25 dilution of the antisera. Ten days after this rabbit was first bled, a boost of 250mg of coupled antigen was given. The following bleed 20 days after the first bleed showed a 20-fold increase in antibody response to the peptide antigen. Forty days after the initial bleed (3rd bleed) a signal of 1.0 OD unit was achieved at a 1:8000 dilution of the antibody, a 16-fold increase in antibody titer over the second bleed. This antibody showed little cross-reactivity with a homologous peptide derived from TGF- β 1 sequences. The TGF- β 1 derived peptide consisted of amino acid numbers 4 to 19 of the mature TGF- β 1 protein. Of the 11 common amino acids, i.e. residues 9-19, 7 are conserved between TGF- β 3 and TGF- β 1.

To determine if the peptide recognizing antibody could recognize TGF- β 3, the antibody was used in Western blot analysis against a trpE:mature TGF- β 3 and trpE:mature TGF- β 1 fusion proteins. As seen in Figure 9, the anti-peptide antibody reacted strongly with the fusion protein of TGF- β 3 while it reacted only weakly with a trpE::TGF- β 1 fusion

protein. Both fusion proteins were recognized by a commercially available anti-TGF- β 1 antibody (R and D systems) (Figure 9).

As can be seen in Figure 9, the anti-peptide antibody recognizing TGF- β 3 also has a high level of background reactivity to bacterial proteins. To reduce this cross reactivity, we purified the antibody on a CNBr-Sepharos column containing the original peptide used as an antigen. The antibody retained its high titer to the peptide of TGF- β 3 and low cross reactivity to the homologous peptide derived from TGF- β 1. The purified antibody reacts very strongly with the fusion protein of TGF- β 3.

Eucaryotic Expression of TGF- β 1 Fused With TGF- β 3

Human recombinant TGF- β 1 has been expressed in monkey COS cells. Sequences encoding the complete precursor of the TGF- β 1 cDNA were cloned downstream from a SV40 promoter using the pSVL eukaryotic expression vector (obtained from Pharmacia). This construct was transfected into COS cells using a standard calcium phosphate precipitation method (13). After transfection, approximately 4×10^6 cells were grown in serum free media for two days. The conditioned media was then collected, acidified and tested for biological activity. Conditioned media from TGF- β 1 transfected cells was found to inhibit the growth of a monolayer mink lung test cell line (CCL 64) by 59% as compared to conditioned media from COS cells transfected with the pSVL vector alone which inhibited growth of CCL 64 cells by only 32%.

A chimeric pro region of the TGF- β 1 precursor::mature TGF- β 3 fusion construct was made by substituting 5' sequences of the TGF- β 1 precursor with sequences encoding TGF- β 3. Given the homology between the two proteins and the conserved position

of their cysteine residues, when such a construct is transfected into COS cells the novel fusion protein is processed into the biologically active mature TGF- β 3. Additional constructs, which consist of the trpE::TGF- β 3 fusion cloned under the regulator sequences of either the SV40 promoter of the long terminal repeat of the mouse mammary tumor virus (MMTV) have been made and tested for biological activity in transient transfection experiments.

EXAMPLE 2: Further sequence determination of a gene encoding
10 **TGF- β 3**

Screening a lambda gt11 human placenta cDNA library (Clontech 1.2×10^6 independent clones) with a repeat free probe of the gene encoding TGF- β 3, resulted in the isolation of a 1.7 kb cDNA clone. On Northern analysis, the mRNA for TGF- β 3 was
15 found to be approximately 3.5 kb, indicating that a full length cDNA had not been obtained.

To obtain additional 5' sequence information, a lambda gt11 human umbilical cord cDNA library (Clontech, 1.5×10^6 independent clones) was screened with a 5' EcoRI-BglII
20 restriction fragment derived from the placenta cDNA clone. This resulted in the isolation of a 1.9 kb cDNA. Sequence analysis revealed this clone contained an additional 180 nucleotides of 5' sequence information. The isolation of this cDNA from an umbilical cord library again confirms that this
25 gene is actively transcribed in this tissue.

To obtain further cDNA sequence information for the gene encoding TGF- β 3, mRNA was isolated from A673 cells and a cDNA library prepared. Starting with 5 μ g poly (A)⁺ RNA, a random primed cDNA library of approximately 2×10^6 clones was
30 constructed in lambda gt10, using the Amersham cDNA synthesis system plus according to the manufacturer's procedures.

Approximately 0.7×10^6 unamplified cDNA clones were screened with a 25-mer oligonucleotide probe (5' ATATAGCGCTGTTTGGCAATGTGCT 3') corresponding to a sequence near the 5' end of the 1.9 kb cDNA clone and a single positive clone containing a 1.7 kb insert was identified.

Analysis of the three overlapping cDNAs (Figure 10) revealed a sequence of 2529 bases, with the largest open reading frame being 1236 bases. No sequence difference were found in the overlapping cDNAs indicating they were derived from transcripts of the same gene. Overlapping sequences comprise a complete 3' untranslated region of 1031 bp with a polyadenylation signal 25 bp upstream from the poly(A) tract. The 5' untranslated region comprises 262 bp but lacks approximately 1 kb, as judged from the size of the mRNA estimated by Northern analysis. The predicted amino acid sequence of the gene encoding TGF- β 3 shows extensive homology to TGF- β 1 and β 2 (Figure 11) (8, 9, 22).

TGF- β 1 and TGF- β 2 are produced in precursor forms of 390 and 414 amino acid residues respectively (8, 9). The cDNA sequence obtained for the gene, i.e. TGF- β 3, encoding TGF- β 3 (Figure 1) contains an open reading frame coding for 412 amino acids, with the first ATG preceded by a stop codon, 162 nucleotides upstream. As found with TGF- β 1 (10), the initiating codon for the protein having tumor inhibitory activity does not form part of a Kozak consensus (20).

Interestingly, six nucleotides downstream there is a second ATG, with an A at position -3, which aligns with the initiating codon in TGF- β 2 (9). Homodimers of the C-terminal 112 residues of TGF- β 1 and β 2 represent the biologically active forms of these proteins. Preceding the site of cleavage to their mature forms, TGF- β 1 and - β 2 have stretches of 4 and 5 basic residues, respectively. In TGF- β 3 there are

5 basic residues preceding the cleavage site marked by the asterisk (Figure 10). The mature forms of TGF- β 1 and - β 2 share 80/112 identical residues. The corresponding 112 C-terminal amino acids in TGF- β 3 exhibit 86/112 and 89/112 5 identical residues compared to TGF- β 1 and - β 2, respectively (Figure 11). Many of the remaining differences represent conservative substitutions. All three proteins show a strict conservation of the cysteine residues in this region. The N-terminal domain of TGF- β 3 precursor exhibits approximately 35% 10 homology to TGF- β 1 and 45% homology to TGF- β 2. By comparison, the corresponding sequences of the TGF- β 1 and - β 2 precursors have 33% sequence homology (Figure 11) (8, 9). Four potential glycosylation sites are contained in the N-terminal part of TGF- β 3 precursor, one of which is contained in all three 15 proteins. All three proteins also possess hydrophobic N-termini which may represent presecretory signal peptide sequences (31). Interestingly, both TGF- β 1 and TGF- β 3 (but not TGF- β 2) contain the fibronectin binding sequence R-G-D (32). By analogy to TGF- β 1 and - β 2, - β 3 is synthesized as a TGF- 20 β 3 precursor which undergoes proteolytic cleavage to produce the mature polypeptide. Based on the functional and structural homology to TGF- β 1 and - β 2, - β 3 likely has therapeutic activity in cancer therapy, wound healing and immunosuppression.

25 EXAMPLE 3: EXPRESSION OF TGF- β 3

TGF- β 3 Expression Construct

A 1500 bp AluI-HgaI restriction fragment of TGF- β 3 cDNA (sites are indicated in Figure 1) which encodes the complete TGF- β 3 protein was cloned into the Bluescript plasmid (Stratagene, La 30 Jolla, CA) to yield the plasmid pBlue-TGF- β 3. The fl intergenic region of this vector allows the production of single stranded DNA via infection of its host bacteria with fl

helper phage. The initiation codon of TGF- β 3 does not form part of a Kozak consensus sequence (CCACC[ATG]G) (20), which has been shown to influence the efficiency of translation. In order to promote high yields of the recombinant TGF- β 3 protein, the flanking sequence of the initiation codon was mutagenized to a more efficient translation sequence by changing CACAC[ATG]A into CCACC[ATG]A using the method of Nakamaye and Eckstein (26). Mutagenesis was confirmed by sequence analysis. Expression yields are further optimized by deletion of TGF- β 3 5' and 3' untranslated [non-coding] sequences. Subsequently, the mutagenized pBlue-TGF- β 3 was cut with KpnI and SpeI, two polylinker restriction sites flanking the cDNA insert. This fragment was cloned into the eukaryotic expression vector pORFEX (3) cut with KpnI and XbaI. In this construct (pCMV:TGF- β 3) the TGF- β 3 cDNA sequence is transcriptionally regulated by the cytomegalovirus immediate early promoter (Figure 12).

DNA Transfection and Gene Amplification

Stable transformants expressing TGF- β 3 were obtained by cotransfecting the pCMV-TGF- β 3 construct (Figure 12) with the dihydrofolate reductase (DHFR) gene (the pDCHIP plasmid containing hamster DHFR minigene driven by its own promoter) into Chinese Hamster Ovary (CHO) cells, which lack the DHFR gene (35).

A standard CaPO_4 DNA precipitation method (13) was used for DNA transfection. pCMV:TGF- β 3 (5.7 kb) and pDCHIP (2.5 kb) were coprecipitated with CaPO_4 in a ratio of 10 μg to 50ng respectively and the precipitate added to 0.5×10^6 CHO(DHFR-) cells. Selection of transformants with a DHFR+ phenotype was performed in alpha MEM (Gibco, Grand Island, NY) supplemented with 10% dialyzed fetal calf serum. The colonies that appeared after culturing for 10-14 days in selection medium

were isolated by standard methods and expanded.

For gene amplification, the primary transfectants were subjected to stepwise selection with increasing concentrations of methotrexate (MTX; Sigma Chemical Co., St. Louis, MO). The first round of selection was carried out at 20nM MTX. TGF- β 3 expression levels were measured by RNA cytodot hybridization normalizing the expression of TGF- β 3 mRNA to that of actin. Two of the three clones with initial high expression (clones CHO 6.35 and CHO 9.1) showed increased TGF- β 3 mRNA expression at 20nM MTX concentration (Figure 13). Total RNA (75 μ g) from CHO cells (lanes 1), CHO 6.35 (lane 2), and CHO 6.35/20nM (lane 3), were fractionated on a 1.2% agarose-formaldehyde gel, blotted onto nitrocellulose and probed with a TGF- β 3 specific probe (EcoRI-SmaI cDNA restriction fragment of a partial TGF- β 3 cDNA clone isolated from umbilical cord; see Figure 10). CHO 6.35/20nM (primary transfectant CHO clone 6.35 at 20nM MTX), which had the highest level of expression, was chosen for initial protein purification from conditioned media and for further gene amplification.

The best clone from further MTX selection (10 μ M MTX) was expanded and a bank of frozen stocks established. This clone, 6.36H, was used in all subsequent production of TGF- β 3 and was maintained in T225 flasks (225 cm²) in alpha MEM supplemented with 10% dialyzed fetal bovine serum. TGF- β 3 production involved seeding Nunc cell factories (6000 cm² of surface area per factory), with cells from three confluent T225 flasks of 6.35H in alpha MEM supplemented with 10% dialyzed FBS. The cells were allowed to grow to 80% confluence in the cell factories. Media was then replaced with HB CHO, a serum-free media from HANA (Hana Biologics). After 72 hours, media was removed and replaced with fresh HB CHO for a total of 5 collections of conditioned HB CHO media. The first collection of conditioned HB CHO media contained low levels of TGF- β 3

with the maximum amounts produced in the 4th through 6th cell collections. Nunc cell factories provide sufficient surface area for the large scale growth of monolayer cell lines such as CHO, yielding a total of 7.5 liters of conditioned media 5 per factor (3 collections, 2.5 liters per collection) with acceptable ease of use in a sterile environment. Using more advanced expression vector systems, it should be possible by one skilled in the art to significantly increase production yields.

- 10 Alternatively, cell lines could be adapted to suspension growth and produced in either a stirred tank fermentation system or in an air lift fermentator. The use of porous glass cylinder supports as a means of adapting monolayer cells (i.e. CHO cells) to stirred or air-lift suspension culture has also 15 been evaluated and shown to give acceptable yields of TGF- β 3.

Expression of a mutant TGF- β 3 Precursor

The mutant TGF- β 3 precursor is expressed as a single homodimeric polypeptide in a host cell by mutation of the R-K-K-R cleavage site between the TGF- β 3 pro region and mature 20 TGF- β 3 to a protease cleavage site, e.g. factor Xa cleavage sequence (Ile-Glu-Gly-Arg) or a collagenase cleavage sequence (Pro-X-Gly-Pro) (Figure 22) using standard site directed mutagenesis procedures, followed by insertion of the mutant TGF- β 3 nucleic acid into an expression vector and transfection 25 of the mutant TGF- β 3/vector DNA into a host cell (e.g. *E. coli*, any mammalian cells, e.g. CHO or HeLa cells, non-mammalian vertebrate cells, e.g. chick cells, and invertebrate cells, e.g. insect cells) together with DNA encoding a selectable marker (e.g. neo, dhfr).

- 30 Additionally, a mutant TGF- β 3 precursor can be produced by preparing DNA comprising a first DNA sequence encoding an

amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG 5 which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by 10 nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising any tri-nucleotide sequence from the list consisting of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT, TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT, CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, 15 ATA, ACT, ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG, GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA, GAG, GGT, GGC, GGA, and GGG. The DNA also comprises a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid 20 sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498. Such DNA may be inserted in a suitable expression vector and transfected into a host cell along with a selectable marker (either linked or unlinked to the 25 expression plasmid) by one ordinarily skilled in the art.

Transfected cells may be cultured in an appropriate medium selecting for cells which express a selectable marker and such cells are further characterized for expression of mutant TGF- β 3. Cells derived in this way may be used to produce mutant 30 TGF- β 3 for subsequent purification. Mature TGF- β 3 may be released from the precursor by proteolytic cleavage when a protease cleavage site separates the mature and the pro region of the TGF- β 3 precursor. Similarly, cyanogen bromide treatment may be used to release mutant, mature TGF- β 3 when

m thi nine separates the mutant, matur TGF- β 3 lacking methionine at nucle tid s 1472-1474 and the pro regi n f the TGF- β 3 precursor.

Biological Assay for Conditioned Media

5 Conditioned media was treated with acetic acid to a final concentration of 0.1 M and serial dilutions tested for biological activity. CCL 64, a cell line derived from Mink lung (American Type Culture Collection, Rockville, MD), was found to be extremely sensitive to the naturally occurring
10 TGF- β 3 isolated from umbilical cord. This cell line was initially chosen, therefore, to test conditioned media for biological activity of the recombinant TGF- β 3 protein according to the method of Iwata, et al. (19). Growth inhibition of CCL 64 mink lung cells produced by TGF- β 1
15 (purified) or TGF- β 3 (from conditioned media) is shown in Figure 14 A/B.

Figure 14A shows a dose response of growth inhibition using purified TGF- β 1 (Calbiochem); a 50% inhibition was obtained with 0.1ng TGF- β 1. An increase in mink cell growth inhibitory
20 activity was found comparing conditioned media form the transfectant selected at 20nM MTX versus media from th parental transfectant. Figure 14B shows the biological activity of acid activated serum free supernatants of CHO 6.35/20 nM transfectant (closed circles) and CHO 6.35
25 transfectant (open circles); 50% inhibition was obtained equivalent to 30 and 5 ng/ml TGF- β 1 activity, respectively. Conditioned medium from parental CHO (DHFR-) possessed much lower growth inhibition than either transfectant. These results clearly show that the TGF- β 3 cDNA is transcribed and
30 that TGF- β 3 mRNA is translated and produces biologically active protein.

In the presence of EGF, acidified conditioned media from CHO 6.35, containing TGF- β 3 was able to promote soft agar growth of NRK cells. Growth of NRK cells in soft agar has been shown to be inducible by stimulating the production of extracellular matrix proteins, an important parameter in wound healing.

Immunodetection

Peptides corresponding to various partial amino acid sequences of the TGF- β 3 protein were synthesized on an Applied Biosystems peptide synthesizer (Model 430A) using tBoc chemistry (Figure 15). Peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and used for immunization of rabbits. Enzyme-linked immunosorbent assays were used initially to characterize the antibody titers (Table 1). For this, and the following immunological experiments, standard techniques were employed (17). High titer antibody from immunized rabbits injected with β 3V or β 3III peptides were purified using an affinity matrix composed of the respective peptide β 3 antigen conjugated to Affi-prep 10 (Bio Rad, Richmond, CA).

20

TABLE 1

Peptide	Sequence	Elisa Titer
I	EEMHGEREEGCTQENTSEY	1:6,000
IIL	GDILENIHEVMEIKRKGVDNEDD	1:10,000
IIs	GDILENIHEVMEIK	1:19,000
25 III	DTNYCFRNLEENC	1:26,000
IV	CVRPLYIDFRQDLGWKWVHEPKGYANFC	1:19,000
V	YLRADTTHTSTVLGLYNTLNPEASAY	1:26,000
VI	CVPQDLEPLTILYYVGRTPKVEQLSNMVKSC	1:4,000

The affinity purified $\beta 3$ III antibody exhibits greater than 300 fold specificity for the $\beta 3$ III peptide compared to the cognate peptide sequences from either the TGF- $\beta 1$ or - $\beta 2$. Furthermore, no significant cross reactivity of this antibody has been observed against either the TGF- $\beta 1$ or - $\beta 2$ proteins. However, this antibody shows only a very limited ability to immunoprecipitate the native recombinant TGF- $\beta 3$ protein from conditioned media. The affinity purified $\beta 3$ V antibody exhibits at least a 400-fold selectivity for the $\beta 3$ V peptide compared to the corresponding peptide sequence from TGF- $\beta 1$. This antibody can also efficiently immunoprecipitate the native TGF- $\beta 3$ protein (Figure 16).

Figure 17 A/B show an immunoblot of TGF- $\beta 3$ in conditioned media produced by the CHO 6.35/20nM transfectant using $\beta 3$ III and $\beta 3$ V antibodies for detection. For peptide blocking experiments, the antibody was preincubated with 80-fold molar excess of peptide prior to incubation with the blot. For detection, alkaline phosphatase (Zymed, San Francisco, CA) conjugated to goat anti-rabbit IgG was used as a second antibody. Figure 17A shows a Western blot of a gel where the sample was subjected to reduction prior to electrophoresis while Figure 17B shows the Western blot of the sample under non-reducing conditions. In each figure, lanes 1-3 and 4-6 corresponds to conditioned media immunoblotted with $\beta 3$ V and $\beta 3$ III antibody, respectively, lanes 2 and 5 immunoblots carried out in the presence of excess cognate peptide, while lanes 3 and 6 represent immunoblots in the presence of an excess unrelated peptide sequence.

Western blotting of conditioned media from CHO 6.35/20nM cells under reducing conditions, using affinity purified $\beta 3$ III and $\beta 3$ V antibody, detected a 50 kDa and a 12 kDa band. These bands correspond to the TGF- $\beta 3$ precursor and mature TGF- $\beta 3$, by analogy to the processing of TGF- $\beta 1$ and TGF- $\beta 2$ described by

Gentry et al. (11) and Madisen et al. (22) (Figure 17A/B).

Under non-reducing conditions, 100 kDa and 24 kDa bands were observed, which we believe to correspond to homodimeric forms of the TGF- β 3 precursor and mature TGF- β 3. The apparent precursor appears as a broad band, characteristic of some glycosylated proteins. Following cleavage of the signal peptide sequence of the precursor form of TGF- β 3, one would expect a protein with MW of 43 kDa (under reduced conditions).

Based on the primary sequence of TGF- β 3, there are four N-linked glycosylation sites, further indicating that the detected precursor protein is glycosylated. Figure 18 A/B show Western blot of cell extract (Figure 18A) and conditioned media (Figure 18B) of the CHO 6.35/20nM transfectant using β 3V antibody for detection. For preparation of cell extracts, cells were first washed with phosphate buffered saline then lysed directly with SDS/ β -mercapthoethanol prior to gel electrophoresis. For peptide blocking (lanes 2 and 4), the antibody was incubated with a 100-fold molar excess of specific peptide prior to incubation with the blot (125 I protein-A was used for detection). In cell extracts of CHO 6.35/20nM under reducing conditions, only the 50 kDa band corresponding to a potential precursor form is detected (Figure 18 A/B). The specificity of the antibody was demonstrated by preabsorbing the antibodies with peptide immunogen prior to Western blotting (Figures 17 A/B and 18 A/B). As expected, based on mRNA and biological activity data, the antibody did not detect any TGF- β 3 protein in conditioned media of the parental CHO (DHFR-) cells.

Both antibody were also tested for immunoprecipitation of native recombinant TGF- β 3 protein (Figure 16). CHO 6.35/20nM were grown to confluency and labeled with [35 S] methionin for 24 hours in methionine-free DMEM in the presence of 5%

dialyzed plus 5% non-dialyzed fetal calf serum. The medium was collected and immunoprecipitated with 10 μ g/ml affinity purified antibody and 20 μ g/ml (1:2 dilution) protein A agarose, for 2 hours at 4°C. Separation of the immunoprecipitated proteins on a 12.5% SDS polyacrylamide gel revealed two proteins migrating identically to the mature TGF- β 3 (12 kDa) and precursor TGF- β 3 (50 kDa) (Figure 16). However, one extra immunoprecipitated protein was found at 43 kDa.

10 The 43 kDa protein may correspond to either the non-glycosylated precursor or a proteolytic breakdown product. The β 3V antibody, in comparison to the β 3III antibody, proved to be much more efficient in immunoprecipitating the TGF- β 3 protein. The specificity of the immunoprecipitation was
15 determined by preincubating the antibody with a 80-fold molar excess of either the cognate peptide or an unrelated peptide sequence. The specific peptide showed complete competition of all three bands whereas the unrelated peptide did not. As expected, based on the amino acid composition and distribution
20 of methionine in the TGF- β 3 protein, the 50 kDa contains significantly more 35 S label.

The β 3V affinity purified antibody was also used in paraffin sections of human umbilical cord (Figures 19 A/B/C/D). Fibroblasts and epithelial cells stained (Figure 19A) as did
25 the smooth muscle fibers of the cord vasculature (Figure 10C) whereas neither the connective tissue nor the extracellular matrix stained with this antibody. A control rabbit polyclonal antibody (Ig against P210^{phl/abl}:OSI catalog #PC02) showed no staining (Figures 19 B and D). The strong staining
30 in umbilical cord tissue agrees with earlier data showing extracts from umbilical cord possessed high levels of mRNA.

Preparation of TGF- β 3 Monoclonal Antibody

A TrpE-TGF- β 3 fusion was produced in E. coli which had the following characteristics, i.e. amino acids 1 to 19 are coded by the TrpE and poly linker segment and amino acids 20 to 170 correspond to amino acids 273 to 412 of the TGF- β 3 precursor (containing the full mature TGF- β 3 sequence). The fusion protein remained in the insoluble fraction after sonication in PBS. Subsequently, the protein was purified by separation on a SDS-polyacrylamide gel and isolated by electroelution. This material was used for immunization of mice by the following protocol:

- a. Balb/C female mice were immunized intraperitoneally with 100 μ g of TrpE-TGF- β 3 in RIBI adjuvant on days 0, 7 and 14;
- b. On day 24 test bleeds indicated high titers against TrpE-TGF- β 3 and purified TGF- β 3 protein;
- c. The mice were then boosted with 100 μ g of the same antigen on days 28, 29 and 30;
- d. Spleen fusions were performed the following day; and
- e. Subsequent methods of hybridoma selection, culture and subcloning were performed following standard procedures (17).

Five stable hybridomas were produced and their characteristics are shown in Table 2. All of the clones produced antibodies of the IgG k class. The monoclonal antibodies immunoblotted with purified TGF- β 3. All five monoclonal antibodies showed no reactivity with TGF- β 1 by ELISA, but crossreacted with TGF- β 2.

Analysis of the epitopes recognized by the monoclonal antibodies using TGF- β 3 synthetic peptides showed that all antibodies reacted with amino acids residues 380 to 412.

**EXAMPLE 4: A METHOD FOR PURIFICATION OF TGF- β 3 FROM
CONDITIONED MEDIA**

Conditioned medium was prepared from CHO 6.35/20nM cells grown to confluence in the presence of 20nM methotrexate. The cells were washed with phosphate buffered saline and incubated with serum free medium for 2 hours to eliminate carryover of serum proteins. Conditioned media was derived from cells incubated with fresh serum-free medium for 48 hours. TGF- β 3 was purified from conditioned media using the following protocol.

- 10 i. Conditioned media was filtered through a 1 μ m glass fiber filter (Micron Separations NC CG 20000-A01) and stored in plastic containers at 4°C after addition of 0.1mM PMSF, 4mM EDTA, 1mM EGTA, 0.02% sodium azide and 10mM Tris HCl pH 7.5.
- 15 ii. Media is concentrated approximately 100 fold using a high capacity, low protein binding Millipore "Pellicon" membrane cartridge (Millipore PLGC Regenerated Cellulose MW cutoff 10,000).
- 20 iii. The protein concentration was adjusted to 10 mg/ml and ammonium sulfate (90%, pH 7) is added to 45%, pH adjusted to 7.6, the material incubated for 4 hours at 0°C (or overnight). The precipitate pelleted by centrifugation (10,000 x g for 30 min) and the pellet left to drain for 10 minutes.
- 25 iv. The pellet was extracted with acetonitrile, 50% (v/v)/acetic acid (1M), at 0°C. 25 ml of extraction buffer was used per gram of starting protein in (iii) above. The suspension was treated to centrifugation at 10,000 x g for 30 minutes.

- v. The extraction supernatant was further concentrated using a Minitan concentrator (Millipore) using identical membranes as described in (ii). 1M acetic acid was added during concentration to prevent protein precipitation and change the buffer to 1M acetic acid.
- vi. The concentrated material is chromatographed by P-60 gel filtration using a 1M acetic acid mobile phase, and peak fractions concentrated using the Minitan concentrator.
- vii. The concentrate was made 1% in Triton X-100, the pH adjusted to 7.5 with solid Tris Base (Sigma) and clarified by centrifugation at 10,000 x g for 30 minutes. This material was then chromatographed (at 4°C) on a β 3V anti-peptide antibody affinity column (12.5 cm x 0.8 cm), the column washed extensively with 0.1M Tris HCl pH 7.5, 10mM EGTA, 1mM PMSF, 1% Triton X-100, 1M NaCl until no protein could be detected in the eluate. TGF- β 3 was then eluted in 50mM glycine, pH 2 into siliconized plastic vials. This material was pooled, acetonitrile was added to a final concentration of 25% and stored frozen at -20°C.
- viii. C18 reverse phase was implemented at this stage prior to in vivo testing. The TGF- β 3 eluted from the antibody affinity column was applied to a Waters C18 reverse phase HPLC column and developed using a 0-60% acetonitrile 0.1% TFA gradient, a flow rate 0.5 ml/minute and monitored using a 210nm flow through detector. Material was aliquoted and stored in the elution solvent at -20°C.

Quantitation of chromatographic yields was accomplished by Western blot using β 3V anti-peptide antibody or by antigen capture assay. The CCL-64 cell bioassay was utilized to test conditioned media and purified chromatographic fractions for biological activity of the recombinant TGF- β 3 protein. The growth inhibition assay is based on measurement of DNA synthesis (18) using the decrease in 5' [125 I]-iodo-2'-deoxyuridine incorporation of treated compared to nontreated cells as a measure for growth inhibition. Conditioned media was acid activated and tested at several dilutions.

Fractions were analyzed by silver stain and Western blot and peak fractions pooled. A silver stained gel identified a single band of 12 kDa and 24 kDa under reducing and non-reducing conditions, respectively (Figure 20). The detection of a single silver staining band indicates that the preparation is greater than 95% homogeneous.

We further describe a novel purification protocol which allows the isolation of the glycosylated precursor form of TGF- β 3 or the pro region of the TGF- β 3 precursor using a lectin affinity column. An example of this is to use commercially available immobilized lectin such as wheat germ agglutinin bound to agarose (e.g. Sigma). The lectin column is washed and equilibrated with 5 column volumes of binding buffer (0.15 M NaCl 50 mM Hepes pH 7.6, 0.1% Triton X-100). Cell lysates or cell conditioned media containing the glycosylated precursor form of TGF- β 3 is suspended in binding buffer and slowly loaded onto the lectin affinity column. It may be necessary to pass the buffer containing TGF- β 3 up to 3 times to maximize binding of the protein to the column. The column is washed with 5 column volumes of the binding buffer to remove unbound material. The column is eluted with binding buffer containing 0.3 M N-acetylglucosamine to elute the bound glycosylated TGF- β 3 precursor from the column. Further, TGF- β 3 is

separated from the other glycoproteins and treated to release the biologically active homodimer from the precursor complex. Other examples of lectins which could be used include but not be limited to ricin, abrin, and Con A.

5 The precursor form of TGF- β 3 or the pro region of the TGF- β 3 precursor may further purified using chromatographic steps known to those skilled in the art. For example, by sequential gel filtration chromatography, ion exchange chromatography, antibody-column chromatography, and high pressure liquid
10 chromatography, or combinations thereof.

Mutants of the TGF- β 3 precursor or mutants of mature TGF- β 3 can be purified using the methods described for the TGF- β 3 precursor and mature TGF- β 3 as described herein.

EXAMPLE 5: ANTIBODIES WHICH NEUTRALIZE TGF- β 3 ACTIVITY

15 Human platelet TGF- β 1 (Collaborative Research, MA), porcine TGF- β 2 (R&D, Minnesota) or purified recombinant human TGF- β 3, at concentrations from 3.125 to 0.049 ng/ml, was incubated with 5 μ g/ml of affinity purified polyclonal rabbit antibodies (β 3V antibody and anti-TGF- β (R&D, Minnesota) for 3 hours at
20 37°C. Control TGF- β 3, TGF- β 2 or TGF- β 1 was incubated without antibodies. Growth inhibition of mink cells by antibody treated and control untreated TGF- β 3, TGF- β 2 or TGF- β 1 was determined as described above. Figures 21A, 21B and 21C show that the β 3V antibody (closed squares) neutralizes the growth
25 inhibitory activity of TGF- β 3, but not TGF- β 2 or TGF- β 1 on mink cells relative to the growth inhibitory activity of identical concentrations of TGF- β 's in the absence of antibody (open circles). Anti-TGF- β (R&D, Minnesota) neutralizes TGF- β 3, TGF- β 2 and TGF- β 1 (Figures 21A, 21B and 21C) (closed
30 circles). Neither antibody had any significant effect on the growth of CCL-64 cells in the absence of TGF- β 3. Antibodies

against the TGF- β 3 peptide β 3V apparently specifically neutralizes the growth inhibitory activity of TGF- β 3.

EXAMPLE 6: BIOLOGICAL CHARACTERIZATION OF TGF- β 3 IN VITRO

Growth was determined using a modification of the monolayer assay for TGF- β 3 described by Iwata, K.K., et al. (19). Non-leukemic cells were subcultured on 96-well tissue culture plates in 100 μ l of media at a seeding density of 2×10^3 cells per well. Cells were maintained and assayed in Dulbecco's modified Eagle's medium containing 10% fetal bovine and 2% L-glutamine. These cells were treated with 25 ng/ml (1 nM) of TGF- β 3, pulsed 24 hours with 1 μ Ci/ml 5-[125 I]-iodo-2'deoxyuridine when cells in the untreated control wells were 90% confluent and harvested.

Leukemic cells (K562, KG-1, KG-1a, HuT 78 and U937) were seeded in 50 μ l of media. K562 was seeded at a density of 1×10^3 cells per well in RPMI supplemented with 10% fetal bovine serum. KG-1 and KG-1a were seeded at a density of 3.5×10^3 cells per well in Iscove's media supplemented with 10% fetal bovine serum. HuT 78 and U937 were seeded at a density of 3.5×10^3 cells per cell in RPMI supplemented with 10% fetal bovine serum. Cell growth was determined by microscopic examination. Examples are shown in Table 2, showing inhibition of some human tumor lines by TGF- β 3.

EXAMPLE 7: DEVELOPMENT OF ANTIGEN CAPTURE ASSAY FOR TGF- β 3

Plates are coated with 50 μ l of affinity-purified rabbit polyclonal antibody (5 μ g/ml in 0.1M NaHCO₃, pH 9.1) made to TGF- β 3 peptide β 3V. Plates were incubated overnight at 4°C. Unbound antibody is removed by aspiration. Plates are blocked with 100 μ l PBS containing 1% BSA (PBS-BSA) for 1 hr at room temperature. The plates are then washed twice with phosphate-

buffer d saline (PBS) containing 0.05% Tween 20 (PBST).

Samples in a final volume of 50 μ l of PBS-BSA are added to the appropriate wells and incubated for 1 hr at room temperature. Unbound protein is removed and the plate is washed four times with PBST. All wells receive 50 μ l of mouse monoclonal antibody against TGF- β 3 (5 μ g/ml in PBS). After incubation for 1 hr at room temperature, unbound antibody is removed and the plate is washed four times with PBST. All wells receive 50 μ l of an appropriate dilution of alkaline phosphatase conjugated to goat anti-mouse antibody. After incubation for 1 hr at room temperature, the plate was washed four times with PBST.

Substrate for alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate) in 100 μ l is added to all of the wells and incubated for 15 min at room temperature. Absorbance in each well measured at 490 nm. Using this assay, we detected between 3-5ng/ml recombinant TGF- β 3.

EXAMPLE 8: EXPRESSION OF CELLULAR AND MEMBRANE-ANCHORED TGF- β 3

Expression of biologically active and inactive TGF- β 3 may be achieved in eukaryotic cells such that the final protein product is retained by the cell and not released into the cell culture media. This has advantages over release of TGF- β 3 into media in concentrating TGF- β 3 to the cell membrane during recovery. Protein sorting of TGF- β 3 within a cell to the endoplasmic reticulum (ER), the plasma membrane or extracellular matrix is achieved through incorporation of specific targeting signals into the TGF- β 3 precursor or mature TGF- β 3 such that targeting signal sequences can be removed

TABLE 2

Effects of TGF- β 3 (1nM) on the Growth of Human Cell Lines in Culture

	<u>CELL LINE</u>	<u>% INHIBITION</u>
5	Human Tumor	
	A549 (lung adenocarcinoma)	46
	A375 (melanoma)	47
	A2058 (melanoma)	88
	WiDR (colon adenocarcinoma)	24
10	MCF 7 (breast carcinoma)	57
	Human Leukemic Cells	
	K562 (CML)	55
	KG-1 (AML)	50
	KG-1a (AML)	50
15	HuT 78 (T cell lymphoma)	50
	U937 (histiocytic lymphoma)	50
	Normal Human	
	Huf (foreskin fibroblasts)	6

during recovery and purification. For example, TGF- β 3 cDNA could be modified so as to produce a TGF- β 3 precursor protein which is membrane anchored via a hydrophobic transmembran sequence. Briefly, such TGF- β 3 proteins are expressed as follows.

The molecular biology techniques use standard methods (23). High level expression vectors for production of TGF- β 3 are constructed with the following properties. Transcription of TGF- β 3 derives from a strong promoter coupled with the
10 enhancers/replication origins for SV40 virus and polyoma virus to further enhance transcription and allow replication in COS cells or polyoma transformed cell lines for short term analysis of the constructs. Expression vectors additionally contain sequences stabilizing the RNA and increasing its half-
15 life (e.g. untranslated sequences from the rabbit β -globin 5' and bovine growth hormone 3' regions), and contain splicing signals to additionally stabilize the RNA transcript (immunoglobulin intron sequences or SV40 small t intron sequences). The vectors contain a Kozak consensus sequence
20 surrounding the initiating methionine to promote efficient translation of the mRNA. TGF- β 2 and TGF- β 3 lack a consensus signal peptide cleavage sequence as defined by Blobel (a basic amino acid, preferably Lys or Arg, followed by an amino acid with a small side chain, commonly Gly or Ala). The TGF- β 3
25 preproprecursor is processed to the precursor form by cleavage of a 22-24 amino acid hydrophobic signal peptide committant to translocation across the endoplasmic reticulum presumably at a cryptic cleavage site. This site can be mutated to a consensus Arg-Ala cleavage sequence using standard site
30 specific mutagenesis techniques to promote optimal secretion/translocation or deleted to promote cytoplasmic localization.

At the carboxyl-terminus of the precursor protein, DNA encoding a hydrophobic transmembrane amino acid sequence (e.g. that encoded by the epidermal growth factor receptor cDNA or c-erbB2 cDNA) followed by a highly charged 'stop transfer' amino acid sequence is inserted such that a TGF- β 3 precursor protein modified in this way binds to the plasma membrane. An amino acid recognition site for a specific protease (e.g. factor X or collagenase) is included between the C-terminal transmembrane sequence and the N-terminal TGF- β 3 precursor to allow efficient cleavage of the TGF- β 3 from the membrane by specific proteases (e.g. factor Xa or collagenase) (Figure 22). An example of this procedure is as follows:

1. A mutation oligonucleotide [5' CTCTGTCGCACGTGGATCCTCAGCTA 3'] is used to engineer a BamHI restriction endonuclease cleavage site 3' of the TGF- β 3 termination codon.
2. A second mutation oligonucleotide is used by one skilled in the art to remove the TGF- β 3 termination codon.
3. Synthetic oligonucleotides are constructed to introduce the protease cleavage site (for example Factor Xa: IleGluGlyArg or collagenase: Pro-X-Gly-Pro) followed by the sequence encoding the c-erbB2 transmembrane sequence and stop transfer sequences (ThrSerIleValSerAlaValValGlyIleLeuLeuValValValLeuGlyValValPheGlyIleLeuIleLysArgArgGlnGlnLysIleArgLysTyrThrMet) such that sequences are inserted in the same translated reading frame as the TGF- β 3 precursor. The nucleic acid molecule produced is inserted in an expression vector and introduced into a host cell line (eg., CHO and HeLa cells).
- Alternatively, linkage of the transmembrane and cytoplasmic domains of vesicular stomatitis virus glycoprotein to the C-

t rminus of the TGF- β 3 precursor with an intervening pr tease cleavage s qu nce (e.g. Factor Xa or collag nas) allows for the production of membrane anchored TGF- β 3 precursor, as described for rat growth hormone (16).

- 5 A number of proteins are anchored to the cell membrane via covalent phosphatidyl inositol linkage (e.g. Qa-2, decay accelerating factor (DAF), Thy-1) (21). Expression of membrane anchored TGF- β 3 may also be achieved by inclusion of phosphatidyl inositol linkage sequences. It is preferable,
10 though not required, that the phosphotidyl inositol linked TGF- β 3 precursor protein may be freed from the membrane by treatment with phospholipase C.

- For example, inclusion of the C-terminal 37 amino acids of DAF (PNKSGTTS GTT RLLSGHTCFTLTGLLGTLVTMGLLT) (4) linked by a Factor
15 Xa protease cleavage sequence (I-D-G-R) or collagenase cleavage sequence (P-X-G-P) to the TGF- β 3 precursor sequence would link TGF- β 3 to the cell in which it is produced by a glycopospholipid membrane anchor attached to the DAF sequence.

- 20 The C-terminal sequences of variant surface glycoprotein (VSG) of Trypanosoma brucei have been shown to be linked to the plasma membrane by a phospholipase C sensitive anchor (21). Linkage of this sequence to C-terminus of the TGF- β 3 precursor via a protease cleavage sequence as previously described
25 enables the synthesis of membrane bound TGF- β 3 precursor protein.

- Alternatively, TGF- β 3 precursor protein may be targeted to the endoplasmic reticulum by deletion of TGF- β 3 signal peptide sequences such that the resulting protein begins with leucine
30 at nucleotide positions 332-334. and by inclusion of an endoplasmic reticulum targeting sequence, for example, the

r tavirus SAII glyc protein VP7 signal sequence (36) int the TGF- β 3 pr curs r sequence. Alternativ ly, the VP7 signal sequence may be linked to the C-terminus of the mature TGF- β 3 such that a protease cleavage sequence (e.g. Factor Xa or 5 collagenase) separates the mature TGF- β 3 from the VP7 signal sequence.

EXAMPLE 9: OPTIMIZATION OF EXPRESSION AND BIOLOGICAL ACTIVITY: STRUCTURE FUNCTION RELATIONSHIPS

TGF- β s exert their biological effects through receptor 10 binding. Three putative receptors have been identified by chemical cross-linking, and are designated type I, type II, and type III receptors with molecular weights 65 kD, 85 kD and ~280 kD, respectively (5, 6). The biological effects of TGF- β 1, -2, and -3 appear to be mediated by type I and/or type II 15 receptors. Cells which lack the type III receptor still respond to TGF- β s; thus, type III receptor binding does not appear essential for at least many of the biological activities of the TGF- β s.

Type III receptor is present at high concentration in the 20 body, relative to type I and II receptors. Type III receptors exists in two forms, a membrane proteoglycan which binds TGF- β via the N-glycosylated core protein and betaglycan, a secreted, soluble form associated with the extracellular matrix (1). Accordingly, it may be possible to mutate TGF- β 3 25 to prevent binding specifically to the type III receptors, thereby increasing the half life in vivo of retained activity.

TGF- β s are also known to bind serum proteins, such as α_2 macroglobulin (29, 18). Thus, binding of TGF- β 3 to α_2 30 macrogl bulin r t other serum pr teins may als sequester,

inactivated or promote clearance of TGF- β 3. TGF- β s are also known to bind the TGF- β pro region and are inactivated in that complex. TGF- β s have a short half-life in vivo (estimated at 2.2 minutes) (7).

5 Degenerate Oligonucleotide Directed Mutagenesis

In order to investigate binding of TGF- β 3 to proteins involved in clearance or inactivation the regions of TGF- β 3 that interact with these proteins (such as α_2 macroglobulin and the secreted form of the betaglycan receptor of TGF- β) are
10 subjected to mutagenesis using degenerate oligonucleotides (5, 25) and, subsequently, recombinant mutant TGF- β 3 are tested for their affinity for such proteins.

The degenerate oligonucleotides spanning the regions involved with binding or clearance proteins are designed such that the
15 3' ends contain an 8 nucleotide palindromic sequence encompassing a restriction endonuclease cleavage site. Preferably, the 5' end of the oligonucleotide consists of sequences encompassing another restriction endonuclease site. The central region (20-100 bp) contains the mutagenized
20 sequence of the TGF- β 3 interaction site. In order to introduce point mutations in the desired regions, the synthesis of the oligonucleotides are programmed such that positions where mutation are not desired (e.g., the restriction sites) are synthesized using solutions of the
25 individual phosphoramidites, while the regions where mutations are desired are synthesized using a defined mixture of phosphoramidites. Using this procedure, a 10% mutation rate can be achieved by a mixture of 90% wild type nucleotide and 3.3% of each of the three "incorrect" nucleotides. Following
30 synthesis, the oligonucleotides are purified either with Sep Pak C18 cartridge for short oligonucleotides (<50 nt) or by denaturing preparative polyacrylamide electrophoresis for

longer oligonucleotides.

The conversion to double-stranded DNA is achieved by self annealing of the oligonucleotides using the 3' (and 5') end palindromic sequences and treatment with DNA polymerase Klenow
5 fragment in the presence of the four dNTPs. After digestion with the restriction enzymes, recognizing the 5' and 3' outside sites, the double stranded oligonucleotide mixture is purified by non-denaturing polyacrylamide gels. Th oligonucleotide mixture is then be used to replace the
10 corresponding fragment in the TGF- β 3 expression plasmid. A double strand sequencing method is used to directly determine the genotype of the mutants.

If the mutation frequency is too low, the mutant clones are identified by differential colony filter hybridization using
15 the kinased 5' ^{32}P labeled oligonucleotide as a probe. The TGF- β 3 mutant expression constructs are transfected into COS cells. Subsequently, the TGF- β 3 protein is partially purified using a TGF- β 3 immunoaffinity column and tested for ability to interact with TGF- β 3 binding/inactivating proteins.

20 Saturation Mutagenesis

In order to create a large number of randomly distributed nucleotide substitutions in the cDNA fragment encoding the complete mature TGF- β 3, a saturation mutagenesis method is applied. The resulting TGF- β 3 mutants are tested for
25 biological activity, receptor binding capacity and affinity for TGF- β binding proteins. Finally, the in vivo half-life of a selected group of mutants is tested.

First the Bluescript (Stratagene) plasmid containing the TGF- β 3 cDNA is subjected to site directed mutagenesis in order to
30 create a FspI site at R-K-K-R processing site of TGF- β 3

(preceding the mature protein) and a BamHI site 3' of TGF- β 3 stop codon. The following mutation oligonucleotides will be used:

5' GTAATTGGTGTCCAATGCGCACTTCTTCCTCTG 3' and

5

┌───┐

FspI

5' CTCTGTCGCACGTGGATCCTCAGCTA 3'

┌───┐

BamHI

10 These sites facilitate removal from and reinsertion of the mutant fragment into the TGF- β 3 cDNA plasmid. The FspI - BamHI TGF- β 3 cDNA target fragment is subcloned in M13 into both orientations relative to a M13 origin of replication.

Three different reactions are performed on each of the strands
15 of the mutagenized target sequence (40 μ g of 1 mg/ml single stranded DNA per tube).

1. 10 μ l of Sodium acetate, pH 4.3 and 50 μ l 2 M sodium nitrite is added and incubated for 60 min at room temperature.
- 20 2. 60 μ l concentrated (18 M) formic acid is added and incubated for 10 min room temperature.
3. 60 μ l of concentrated (12 M) hydrazine is added and incubated for 10 min at room temperature.

After incubation, DNA's are ethanol precipitated, washed with
25 70% ethanol and resuspended in TE buffer. The 2nd strand are prepared by primer extension using AMV reverse transcriptase (depurination inhibits DNA synthesis by E. coli DNA pol I but

n t by reverse transcriptase). Mutants can be identified directly by DNA sequencing f rand m clones.

Binding of TGF- β 3 to α_2 -macroglobulin

The binding of α_2 -macroglobulin to TGF- β 3, modified TGF- β 3 or
5 TGF- β 3 bound to anti-TGF- β 3 antibody is measured using a
modification of the method described by O'Conner-McCourt, et
al. (29). Briefly, ^{125}I TGF- β 3 is incubated with α_2 -
macroglobulin in PBS with either unlabeled TGF- β 3, modified
TGF- β 3 or TGF- β 3 bound to anti-TGF- β 3 antibody for 5 hr on
10 ice. Non-specific binding will be determined using a 400-fold
molar excess of unlabeled growth factor. Crosslinking of th
 α_2 -macroglobulin to the ^{125}I TGF- β 3 is accomplished with the
addition of a $\frac{1}{4}$ volume of 5 mM bis(sulfosuccinimidyl)suberat
(BS³; Pierce) in PBS and the reaction is stopped after 2 min
15 at 4°C by the addition of 1/20 volume of 2.5 M glycine. An
equal volume of SDS-PAGE sample buffer (2X) is added to the
sample will be heated in a boiling water bath for 3 min.

Electrophoresis is performed on the samples and destained gels
is dried and exposed at -70°C to X-ray film using intensifying
20 screens. Alternatively, commercially available anti- α_2 -
macroglobulin (Sigma) is used to immunoprecipitate the α_2 -
macroglobulin ^{125}I TGF- β complex with or without crosslinking
and quantitated directly by a gamma counter.

Binding assay of TGF- β 3 to type III receptors

25 Type III receptors exist in both a membrane bound
(proteoglycan) and soluble (betaglycan) forms. The binding of
betaglycan to TGF- β , modified TGF- β or TGF- β bound to anti-
TGF- β antibody is measured using the method described by
Andres, et al (1). Non-specific binding is determined using
30 a 400-f ld m lar excess of unlabeled growth factor. Briefly,

soluble betaglycan is made from serum-free conditioned media from 3T3-L1 adipocytes. Mouse 3T3-L1 adipocytes is grown to confluence or near confluency. The cell monolayers are washed twice and incubated for 3 days in serum-free Waymouth's 5 medium. A concentrated stock of fresh PMSF is added to a final concentration of 0.2 mM immediately after collection. The collected conditioned media is centrifuged at 2,000 x g for 15 min at 4°C followed by ultracentrifugation at 2000,000 x g for 40 min at 4°C. NaCl is added to the conditioned media 10 to a final concentration of 0.25 M NaCl and brought to pH 6.0 by the addition of 1 M BIS-Tris, pH 6.0 and pre-equilibrated with binding buffer (50 mM NaCl, 10 mM MgCl₂, 5 mM KCl, 25 mM Hepes, pH 7.5 containing 1 mg/ml of BSA). ¹²⁵I TGF-β is mixed with incubated with unlabeled TGF-β, modified TGF-β or TGF-β 15 bound to anti-TGF-β antibody and soluble betaglycan (appropriately diluted) in binding buffer. This mixture is added to the pre-equilibrated DEAE-Sepharose Fast Flow and incubated for 3.5 hours at 4°C with continued mixing. The beads are then washed 5 times with cold binding buffer 20 (without albumin). The amount of radioactivity remaining on the beads will be quantitated. For analysis of specific binding to the type III proteoglycan receptors, ¹²⁵I TGF-β3 is cross-linked to intact cells (6), the receptors separated by standard SDS-PAGE, and ¹²⁵I-binding quantitated by 25 densitometry.

Pharmacokinetics of TGF-β3

The chemical half-life of TGF-β3 is determined in the serum of mice following bolus injections (0.1-10μg/mouse) via i.v., i.p., and s.c. routes, using internally labelled TGF-β3 30 (labelled metabolically with ³⁵S cysteine) or ¹²⁵I TGF-β3. Tissue distribution of labelled material is measured in various organs with particular emphasis on liver, spleen and bone marrow sites. If the biological half-life of TGF-β3 in

vivo is found to be unacceptably short regional administration by direct intrasplenic injection (through the body wall) is employed or using the surgical technique reported by Goey et al (12) involving injection into the femoral artery. This latter approach has been reported to be effective in localizing TGF- β 1 to the marrow with resulting inhibition of early stem cell and progenitor cell proliferation.

Example 10: Expression of a Pro Region of the TGF- β 3 Precursor

The TGF- β 3 pro region protein associates with the mature TGF- β 3 and modifies the half life and biological activity of mature TGF- β 3. Nucleic acid encoding the TGF- β 3 precursor beginning with methionine at nucleotide positions 263-265 and ending with arginine at position 1160-1162 is engineered by mutagenesis of the nucleic acid in Figure 1 to introduce a translation termination codon (TGA, TAG, TAA) at position 1163-1165. The resulting nucleic acid is inserted in an expression vector and transfected into a suitable host cell with an additional selectable marker, as previously described. TGF- β 3 pro region protein is recovered from the culture medium. This protein stably binds mature TGF- β 3 and thereby sequesters and modifies the half life and biological activity of the mature TGF- β 3.

Binding assay of TGF- β 3 to the TGF- β 3 pro region protein

The binding of TGF- β 3 pro region to TGF- β 3 or mutant TGF- β 3 is measured by the following. ^{125}I TGF- β 3 is incubated with purified TGF- β 3 pro region in PBS with either unlabeled TGF- β 3 or mutant TGF- β 3 for 5 hr on ice. Non-specific binding will be determined using a 400-fold molar excess of unlabeled growth factor. Crosslinking of the TGF- β 3 pro region to ^{125}I TGF- β 3 is accomplished with the addition of a $\frac{1}{4}$ volume of 5 mM bis(sulfosuccinimidyl)suberate (BS^3 ; Pierce) in PBS and the

reaction is stopped after 2 min at 4°C by the addition of 1/20 volume of 2.5 M glycine. An equal volume of SDS-PAGE sample buffer (2X) is added to the sample will be heated in a boiling water bath for 3 min.

5 Electrophoresis is performed on the samples and destained gels is dried and exposed at -70°C to X-ray film using intensifying screens. Alternatively, anti-TGF- β 3 is used to immunoprecipitate the TGF- β 3 pro region complexed with ^{125}I TGF- β complex with or without crosslinking and quantitated 10 directly by a gamma counter.

REFERENCES

1. Andres, J.L., et al. (1989) J. Cell Biol. 109:3137-3145.
2. Assoian, et al. (1983) J. Biol. Chem. 258: 7155.
3. Barker, C.R., Worman, C.P., and Smith, J.L (1975)
5 Immunology 29:765-777.
4. Caras et al. (1989) J. Cell Biol. 108:1387-1396.
5. Cheifetz, S., et al. (1988) J. Biol. Chem. 263:16984-16991.
6. Cheifetz, S. et al. (1987) Cell 48:409-415.
- 10 7. Coffey, R.J. et al. (1987) J. Clin. Invest. 80:750-757.
8. R. Derynck et al. (1985) Nature, 316:701.
9. de Martin, et al. (1987) EMBO J 6:3673-3677.
10. Derynck et al. EMBO J. 6:3673-3677.
11. Gentry, L.E. et al. (1987) Mol. Cell. Biol. 7:3418-3427.
- 15 12. Goey, H. et al.(1989) J. Immunol. 143:877-880.
13. Graham, F.L. and van der Eb, A.J. (1973) Virology 52:456-457.
14. Jeannette L. Graycar, et al. (1989) Mol. Endo., 3(12):1977.

15. Grosveld, t al. (1981) Gene 13: 227-237.
16. Guan, X.-P. et al. (1989) Cancer Comm. 1:111-115.
17. Harlow, E. and Lane D. (1988) In Antibodies, A Laboratory Manual, Cold Spring Harbor.
- 5 18. Huang, S.S. et al. (1988) J. Biol. Chem. 263:1535-1541.
19. Iwata, K.K. et al. (1985) Cancer Res. 45:2689-2694.
20. Kozak, M. (1986) Cell 44:283-292.
21. Low (1989) Biochem. Biophys. Acta. 988:427-454.
22. Madisen, L. et al. (1989) DNA 8:205-212.
- 10 23. Maniatis, T. et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press.
24. Massague, J. (1987) Cell, 49, 437-438.
25. Matteucci, M.D. and Heyneker, H.L. (1983) Nucl. Acids Res. 11:3113-3121.
- 15 26. Nakamaye, K. and Eckstein, F. (1986) Nucleic Acids Res. 14:9679-9698.
27. Neuberger, et al. (1984) Nature (London), 312:604.
29. Neuberger, M.J. et al. (1985) Nature (London), 314:265-270).
- 20 29. O'Connor-McCourt, M. and Wakfield, L.M. (1987) J. Biol. Chem. 262:14090-14099.

30. Oi, V.T., et al. (1986) BioTechniques 4:21.
31. Perlman and Halvorson (1983) J. Mol. Biol. 107:391-409.
32. Rucslahti and Pierschbacher (1986) Cell 44:517-518.
33. Sanger, et al. (1977) Proc. Natl. Acad. Sci. 74: 5463-5467.
34. Southern, (1975) J. Mol. Biol. 98: 503-517.
35. Urlaub, G. and Chasin, A. (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220.
36. Whitfeld, et al. (1987) Mol. Cell. Biol. 7:2492.
- 10 37. Wrann, M. et al. (1987) EMBO J. 6:1633-1636.
38. U.S. Patent No. 4,886,747, issued December 12, 1989 to R. Derynck and D. Goeddel.

What is claimed is:

1. A method for recovering purified, non-denatured mature TGF- β 3 from a mixture of mammalian cell-derived polypeptides which comprises contacting the mixture with
5 an antibody which specifically binds to mature TGF- β 3 but exhibits substantially no cross reactivity with mature TGF- β 1 and mature TGF- β 2.
2. The method of claim 1, wherein the mixture of mammalian cell-derived polypeptides includes a mixture of non-human
10 mammalian polypeptides from non-human cells in which TGF- β 3 has been expressed.
3. The method of claim 1, wherein the antibody is directed to an epitope defined by the amino acid sequence YLRSDTTHSTVLGLYNTLNPEASASY.
- 15 4. The method of claim 3, wherein the antibody is immobilized on a solid support.
5. A method for producing substantially purified TGF- β 3 precursor having an anchorage membrane sequence which comprises:
20 (a) preparing DNA encoding a TGF- β 3 precursor having the membrane anchorage sequence;
(b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;
(c) transforming the host cell with the vector in order
25 to induce expression of the DNA of step (b) such that a TGF- β 3 precursor is expressed and subsequent translocation of the expressed TGF- β 3 precursor having the membrane anchorage sequence ;
(d) culturing the host cell in medium;

- (e) separating the host cell from the medium;
 - (f) disrupting the host cell such that a lysate containing the TGF- β 3 precursor having the membrane anchorage sequence is produced; and
 - 5 (g) purifying the TGF- β 3 precursor having a membrane anchorage sequence from the lysate under conditions such that the substantially purified TGF- β 3 precursor is produced.
6. A method for producing substantially purified TGF- β 3 precursor having an anchorage membrane sequence which comprises:
- 10 (a) preparing DNA encoding the TGF- β 3 precursor having a membrane anchorage sequence;
 - (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;
 - 15 (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a TGF- β 3 precursor is expressed and subsequent occlusion of the expressed TGF- β 3 precursor having a membrane anchorage sequence in occlusion bodies;
 - 20 (d) culturing the host cell in culture medium;
 - (e) separating the occlusion bodies from the host cells and the culture medium;
 - (f) disrupting the occlusion bodies to produce a solution containing the TGF- β 3 precursor having a membrane anchorage sequence; and
 - 25 (g) purifying the TGF- β 3 precursor having a membrane anchorage sequence from the lysate such that the substantially purified TGF- β 3 precursor is
 - 30 produced.
7. The method of any of claims 5 or 6, wherein the anchorage linkage sequence includes a phosphatidyl inositol linkage.

8. The method of any of claims 5 or 6, wherein the anchorage linkage sequence includes a hydrophobic transmembrane peptide sequence.
9. A method for producing a substantially purified mutant TGF- β 3 precursor which comprises:
- 5 (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498;
- 10 (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;
- 15 (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF- β 3 precursor is expressed;
- 20 (d) culturing the host cell in a medium under conditions such that the expressed mutant TGF- β 3 precursor is secreted into the medium;
- 25 (e) separating the cell from the medium containing the mutant TGF- β 3 precursor so secreted; and
- 30 (f) purifying the mutant TGF- β 3 precursor thereby producing the substantially purified mutant TGF- β 3 precursor.

10. A method for producing a substantially purified mutant TGF-83 precursor which comprises:
- 5 (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamin encoded by nucleotides 1148-1150, a second DNA sequence which is linked to nucleotide 1150 encoding a protease cleavage sequence, and a third
10 DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with a serine encoded by nucleotides 1496-1498;
 - (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;
 - 20 (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) such that a mutant TGF-83 precursor is expressed;
 - (d) separating the host cell containing the expressed, mutant TGF-83 precursor from the culture medium;
 - 25 (e) disrupting the host cell such that a lysate containing the mutant TGF-83 precursor is produced; and
 - (f) purifying the mutant TGF-83 precursor thereby producing the substantially purified mutant TGF-83
30 precursor.
11. A method for producing a substantially purified mutant TGF-83 precursor which comprises:
- (a) preparing a DNA comprising a first DNA sequence

- encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498;
- (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;
- (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a mutant TGF- β 3 precursor is expressed;
- (d) culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 precursor is secreted into the medium;
- (e) separating the cell from the culture medium containing the mutant TGF- β 3 precursor so secreted; and
- (f) purifying the mutant TGF- β 3 precursor such that a substantially purified mutant TGF- β 3 precursor is

produced.

12. A method for producing a substantially purified mutant TGF- β 3 precursor which comprises:

- 5 (a) preparing a DNA comprising a first DNA sequence encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a methionine encoded by nucleotides 263-265 and ending with a glutamine encoded by nucleotides 1148-1150, a second DNA
10 sequence comprising ATG which is linked to nucleotide 1150, a third DNA sequence, linked to the second DNA sequence, encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with an
15 alanine encoded by nucleotides 1163-1165 and ending with an asparagine encoded by nucleotides 1469-1471, a fourth DNA sequence, linked to the third DNA sequence, comprising X, and a fifth DNA sequence, linked to the fourth DNA sequence,
20 encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a valine encoded by nucleotides 1475-1477 and ending with a serine encoded by nucleotides 1496-1498;
- 25 (b) inserting the DNA of step (a) into an expression vector linked to a suitable promoter compatible with a host cell;
- (c) transforming the host cell with the vector in order
30 to induce expression of the DNA of step (b) under conditions such that a mutant TGF- β 3 precursor is expressed;
- (d) separating the host cell containing the expressed, mutant TGF- β 3 precursor from the culture medium;
- () disrupting the host cell such that a lysate

containing the mutant TGF- β 3 precursor is produced;
and

(f) purifying the mutant TGF- β 3 precursor such that a
substantially purified mutant TGF- β 3 precursor is
produced.

5

13. The method of any of claims 11 or 12, wherein in step (a)
X is selected from a group of tri-nucleotides consisting
of TTT, TTC, TTA, TTG, TCT, TCC, TCA, TCG, TAT, TAC, TGT,
TGC, TGG, CTT, CTC, CTA, CTG, CCT, CCC, CCA, CCG, CAT,
CAC, CAA, CAG, CGT, CGC, CGA, CGG, ATT, ATC, ATA, ACT,
ACC, ACA, ACG, AAT, AAC, AAA, AAG, AGT, AGC, AGA, AGG,
GTT, GTC, GTA, GTG, GCT, GCC, GCA, GCG, GAT, GAC, GAA,
GAG, GGT, GGC, GGA, and GGG.

10

14. The method of any of claims 5, 6, 9, 10, 11, or 12,
wherein purification includes affinity chromatography.

15

15. The method of any of claims 5, 6, 9, 10, 11, or 12,
wherein affinity chromatography comprises lectin column
chromatography.

16. The method of claim 14, wherein affinity chromatography
comprises antibody column chromatography.

20

17. The method of any of claims 9 or 10, wherein in step (a)
the protease cleavage sequence includes a collagenase
recognition sequence.

18. The method of any of claims 9 or 10, wherein in step (a)
the protease cleavage sequence includes a Factor Xa
recognition sequence.

25

19. The method of any of claims 5 or 6 which further
comprises:

- (a) treating the purified TGF- β 3 precursor s recovered with an activating agent to separate a mature TGF- β 3 from the precursor; and
 - (b) recovering the separated mature TGF- β 3 of step (a).
- 5 20. The method of any of claims 9 or 10 which further comprises:
 - (a) treating the purified mutant TGF- β 3 precursor so recovered with an activating agent to separate a mature TGF- β 3 from the precursor; and
 - 10 (b) recovering the separated mature TGF- β 3 of step (a).
- 21. The method of any of claims 11 or 12 which further comprises:
 - (a) treating the purified mutant TGF- β 3 precursor s recovered with cyanogen bromide to separate a mature TGF- β 3 from the precursor; and
 - 15 (b) recovering the separated mature TGF- β 3 of step (a).
- 22. A method for producing mutant TGF- β 3 which comprises:
 - (a) preparing a DNA encoding an amino acid sequence substantially identical to the amino acid sequence shown in Figure 1 beginning with a leucine encoded by nucleotides 332-334 and ending with a serine encoded by nucleotides 1496-1498;
 - 20 (b) inserting the DNA of step (a) into an expression vector operably linked to a suitable promoter compatible with a host cell;
 - 25 (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a mutant TGF- β 3 is expressed;
 - (d) culturing the host cell in medium;
 - 30 (e) separating the host cell containing the mutant TGF-

B3 is expressed from the medium;

- (f) disrupting the cell to produce a lysate containing the mutant TGF- β 3; and
- (g) purifying the mutant TGF- β 3.

5 23. The method of claim 22 which further comprises:

- (a) treating the purified mutant TGF- β 3 so recovered with an activating agent to separate a mature TGF- β 3 from the mutant TGF- β 3; and
- (b) recovering the separated mature TGF- β 3 of step (a).

10 24. A process which comprises:

- (a) contacting a TGF- β 3 precursor with a precipitating agent thereby concentrating the TGF- β 3 precursor in a precipitate;
- (b) extracting the pellet of step (a) with an acidified
15 organic solution under such conditions that mature TGF- β 3 is separated from the pellet; and
- (c) recovering the mature TGF- β 3 so separated in step (b).

20 25. The process of claim 24, wherein the acidified organic solution in step (b) includes acidified acetonitrile.

26. The process of claim 25, wherein the acidified acetonitrile comprises 50% acetonitrile and 1.0M acetic acid.

25 27. The process of claim 24, wherein the precipitating agent in step (a) includes ammonium sulfate.

28. A method for producing and identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins which comprises:
(a) preparing a DNA encoding the TGF- β 3;

- (b) performing mutagenesis on the DNA of step (a) thereby obtaining a mutant DNA;
- (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell;
- 5 (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF- β 3 is expressed;
- 10 (e) culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 is secreted into the medium;
- (f) separating the host cell from the culture medium containing the mutant TGF- β 3 so expressed;
- 15 (g) purifying the mutant TGF- β 3;
- (h) activating the mutant TGF- β 3 so expressed under conditions such that a mutant mature TGF- β 3 is separated from the mutant TGF- β 3; and
- (i) assaying the culture medium for the mutant, mature TGF- β 3 thereby identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins.
- 20
29. A method for producing and identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins which comprises:
- 25 (a) preparing a DNA encoding the TGF- β 3;
- (b) performing mutagenesis on the DNA of step (a) thereby obtaining a mutant DNA;
- (c) inserting the mutant DNA into an expression vector linked to a suitable promoter compatible with a host cell;
- 30 (d) transforming the host cell with the vector in order to induce expression of the mutant DNA of step (c) under conditions such that a mutant TGF- β 3 is

express d;

- () culturing the host cell in medium under conditions such that the expressed mutant TGF- β 3 is produced in the host cell;
- 5 (f) separating the host cell containing the mutant TGF- β 3 so expressed from the culture medium;
- (g) disrupting the cells to produce a lysate containing the mutant TGF- β 3;
- (h) purifying the mutant TGF- β 3;
- 10 (i) activating the mutant TGF- β 3 so expressed under conditions such that a mature TGF- β 3 is separated from the mutant TGF- β 3; and
- (j) assaying the culture medium for the mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins thereby identifying a mutant, mature TGF- β 3 exhibiting reduced binding affinity to serum binding proteins.
- 15

30. The method of any of claims 28 or 29, wherein the serum binding protein is α 2-macroglobulin.

20 31. The method of any of claims 28 or 29, wherein the serum binding protein is type III TGF- β receptor.

32. The method of claim 31, wherein the type III TGF- β receptor is betaglycan.

25 33. The method of any of claims 28 or 29, wherein the serum binding protein is a pro region of the TGF- β precursor.

34. A method for producing a substantially purified pro region of the TGF- β 3 precursor which comprises:

- (a) preparing DNA encoding the pro region of the TGF- β 3 precursor;
- 30 (b) inserting the DNA into an expression vector linked

- to a suitable promoter compatible with a host cell;
- 5 (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF- β 3 precursor is expressed;
- (d) culturing the host cell in medium;
- (e) separating host cells from the medium;
- 10 (f) disrupting the host cells to produce a lysate containing the pro region of the TGF- β 3 precursor so expressed; and
- (g) purifying the pro region of the TGF- β 3 precursor from the lysate such that the substantially purified pro region of the TGF- β 3 precursor is produced.
- 15 35. A method for producing a substantially purified pro region of the TGF- β 3 precursor which comprises:
- (a) preparing DNA encoding the pro region of the TGF- β 3 precursor;
- 20 (b) inserting the DNA into an expression vector linked to a suitable promoter compatible with a host cell;
- (c) transforming the host cell with the vector in order to induce expression of the DNA of step (b) under conditions such that a pro region of the TGF- β 3 precursor is expressed;
- 25 (d) culturing the host cell in medium under conditions such that the expressed pro region of the TGF- β 3 precursor is secreted into the medium;
- (e) separating host cells from the medium containing the pro region of the TGF- β 3 precursor so secreted;
- 30 and
- (f) purifying the pro region of the TGF- β 3 precursor such that the substantially purified pro region of the TGF- β 3 precursor is produced.

36. The method of any of claims 5, 6, 9, 10, 11, 12, 22, 28, 29, 34, or 35, wherein the host cell is a eucaryotic cell.
37. The method of any of claims 5, 6, 9, 10, 11, 12, 22, 28, 29, 34, or 35 wherein the host cell is a procaryotic cell.

1/29

FIGURE 1

CCAGCAAACCTGTTTAGACACATGGACAAGAAATCCAGCGCTACAAGGCACACAGTCGGCTTCTTCGTCCTCAGGGTTGCCAGCGCTTCTTGGAAAGTCC
 10 20 30 40 50 60 70 80 90 100
 TGAAGCTCTCGCAGTGCAGTTCATGCACCTTCTTGGCAAGCCCTCAGTCTTGGGAATCTGGGAGCGCGCTGGTTTCTTCCCTCCTTCTTGCACGT
 110 120 130 140 150 160 170 180 190 200
 CTGCTGGGGTCTCTTCTTCCAGGCTTGGCGTCCCTTGGCTCTCTTCCAGCTCACACATGAAGATGCACCTTGCAGAGGGCTCTGGTGGTCTCTGGC
 210 220 230 240 250 260 270 280 290 300
 L L N F A T V S L S L S T C T T L D F G H I K K K R V E A I R G Q
 CCTGCTGAACCTTGGCCAGGTCAGCTCTCTCTGTCACCTTGCACCCACTTGGACTTCGGCCACATCAAGAAGAGGGTGGAGGCCATTAGGGGACAG
 310 320 330 340 350 360 370 380 390 400
 I L S K L R L T S P P E P T V M T H V P Y Q V L A L Y N S I R E L
 ATCTTGAGCAAGCTCAGGCTCACCAGCCCCCTGAGCCCAACGGTGAATGACCCACGCTCCCTATCAGGTCTTGGCCCTTTTACACAGCACGCCCGGAGCTGC
 410 420 430 440 450 460 470 480 490 500
 L E E M H G E R E E G C T Q E N T E S E Y Y A K E I H K F D M I Q G
 TGGAGGAGATGCATGGGGAGAGGAGGAGGCTGCACCCAGGAAACACCGAGTGGGAATACTATGCCAAAGAAATCCATAATTCGACATGATCCAGGG
 510 520 530 540 550 560 570 580 590 600
 L A E H N E L A V C P K G I T S K V F R F N V S S V E K N R I N L
 GCCTGGCGGAGCACAACTGGCTGTCTGCCCCCTAAGGAATTACCTTCCAGGTTTTCGGCTTCAATGTGCTTCCAGTGGAGAGAAATAGAACCAACCTA
 610 620 630 640 650 660 670 680 690 700
 F R A E F R V L R V P N P S S K R N E Q R I E L F Q I L R P D E H
 TCCGAGCAGAAATCCGGGCTTGGGGTGGCCCAACCCAGCTCTAAGCGGAATGACGAGGATCGAGCTCTCCAGATCTTCGGCCAGATGAGCACA
 710 720 730 740 750 760 770 780 790 800
 I A K Q R Y I G G K N L P T R G T A E W L S F D V T D T V R E W L L
 TGGCCAAACAGCGCTATATCGGTGGCAAGAAATCGCCACACGGGGCACTGCCGAGTGGCTGTCTTGTGATGTACTGACACTGTGCGTGGAGTGGCTGTT
 810 820 830 840 850 860 870 880 890 900
 R R E S N L G L E I S I H C P C H T F Q P N G D I L E N I H E V M
 GAGAAGAGAGTCCAACTTAGGCTTAGAAATCAGCATTCACCTGTCCATGTACACCTTTCAGCCCAATGGAGATATCTTGGAAACATTCACGAGGTGATG
 910 920 930 940 950 960 970 980 990 1000
 E I K F K G V D N E D D H G R G D L G R L K K Q K D H H N P H L I
 GAAATCAAATTCAAAGGCTGGACAAATGAGGATGACATGGCCGTGGAGATCTGGGGCGCTCAAGAAGCAGAGGATCACCACACCCCTCATCTAATCC
 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
 L M M I P P H R L D N P G Q G G Q R K K R A L D T N Y C F R N L E E
 TCAITGATATCCCCCACACCGGCTCGACAACCCGGGCGGGGTTCAGAGGAAGAGCGGGCTTGGACACCAATTAATGCTTCCGCACTTGGAGGA
 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 N C C V R P L Y I D F R Q D L G W K W V H E P K G Y Y A N F C S G
 GAATGCTGTGGCGCCCCCTTACATTCAGTTCAGGATCGGCTGAGGCTTCCATGAGCTAAGGGCTACTATGCCAACTTCTGCTCAGGC
 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300

2/29

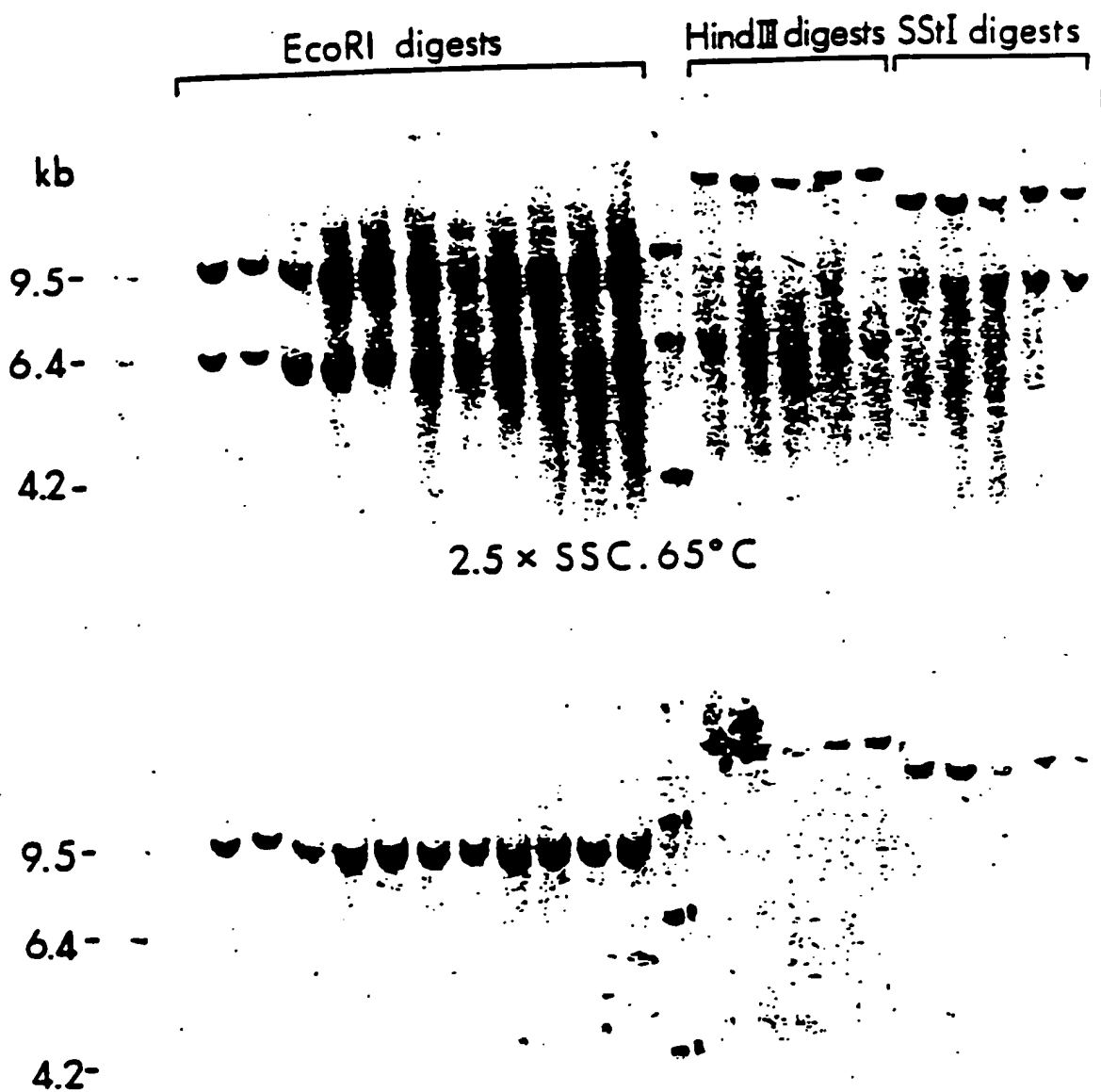
FIGURE 1

continued

P C P Y L R S A D T T H S T V L G L Y N T L N P E A S A S P C C V
 CCTTGGCCATACCTCGCAGTGCAGACACACCCACAGCAGGTGCTGGGACTGTACAACTCTGAACCTGAAGCATCTGCCTTGGCTGGTGC 1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
 P Q O L E P L T I L Y Y V G R T P K V E Q L S N M V V K S C K C S
 CCCAGGACCTGGAGCCCTGACCATCTGTACTATGTTGGGAGGACCCCAAGTGGAGCAGCTCTCCAACAATGGTGGTGAAGTCTTGTAAATGTAGCTG 1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
 AGACCCACGTCGACAGAGAGAGGGGAGAGAGAACCCACTGCTGACTGCCGCTCCTCGGGAACACACAAGCAACAACCTCACIGAGAGGCCCTG 1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
 GAGCCCAACAACCTCGGCTCCGGGCAATGGCTGAGATGGAGGTTCTTTTGGAACTTTCTTTCGCTGCTGAGAAATCACGGTGGTAAAGAAAG 1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
 TGTGGGTTGGTTAGAGGAAGGCTGAACCTCTCAGAACACACAGACTTCTGTGACGCACACAGAGGGGATGGGATAGAGGAAGGATGGTAAAGTTGA 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 CATGTTGTGGCAATGGGATTGGGCTACCCCTAAAGGGGAGAGGAAGGCGCAGAGAAATGGCTGGGTGAGGCGCAGACTGGAAGACACTTCAGATCTGAGG 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
 TTGGATTGGCTCATTTGCTACCATCTGCTCTAGGGAATCTGGATTATGTTATACAAAGGCAAGCAATTTTTTTTTTAAAGACAGGTACGAAGA 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
 CAAAGTCCAGAAATTGTATCTCATATCTGCTGCTGGGATTAAGGGCAATCTATTACTTTTGCAAACTGCTCTACATCAATTAACATCGTGGGTCACTACA 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 GGGAGAAAATCCAGGTTCATGCAGTTCCTGGGCCCATCAACTGATGTTGGGCTTTTGGATATGCTGAACGCAGAGAAGGGTGGAAATCAACCCCTCTCCTG 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
 TCTGCCCTCGGCTCCCTCCCTCCCTCGATCATATTTCCCTTGGACACTTGGTAGACGCCCTCCAGGTGAGGATGCACATTTCTGGATTGT 2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
 GGTTCACAGCAGGGTTGGGGCATTAATGGGTTCTTCCCTCCCTCCCTCCCTGTTTCAATTTGGTGTTCCTGGAGCAGGTGCGACAACATGTG 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 AGGCATTCGGGGGAAGCTCGACATGTCGACACAGTGTGGCCCCAGACGCTAGAGGTAAAGACAAGTATGAATATTACTCTCAAAATCTTT 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
 GTATAAAIAAAATTTTGGGGCAATCTCTG poly(A) 2510 2520

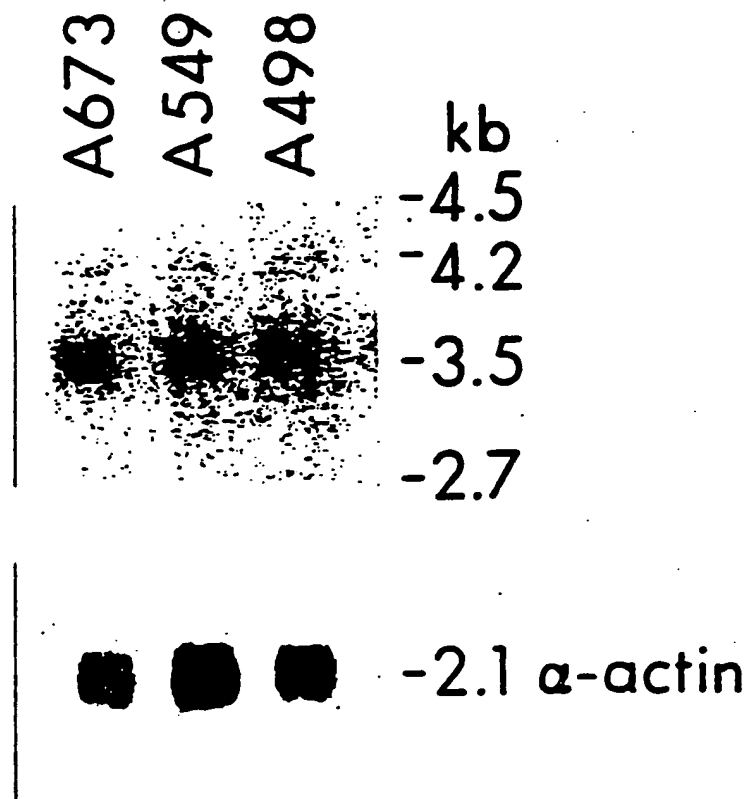
3/29

FIGURE 2



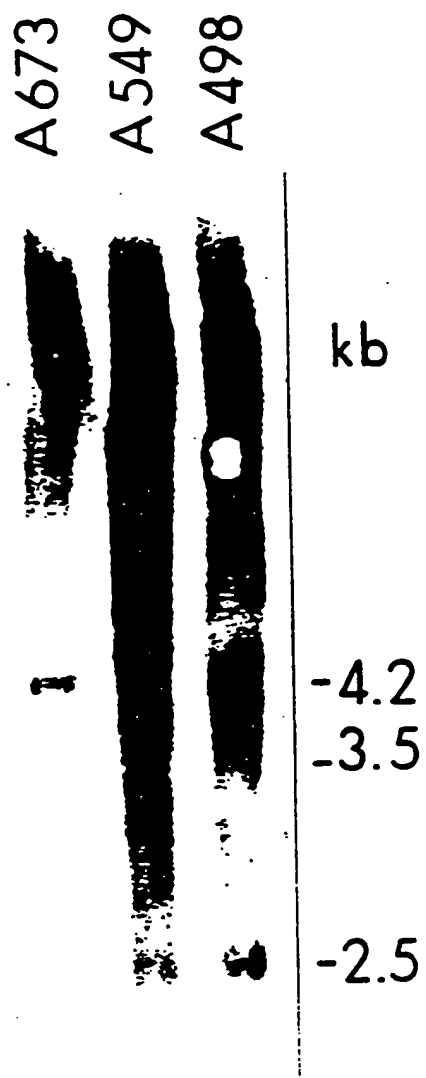
4/29


FIGURE 3



5/29

FIGURE 4



 -2.1 α -actin

6/29

FIGURE 5

A673
A549
A498

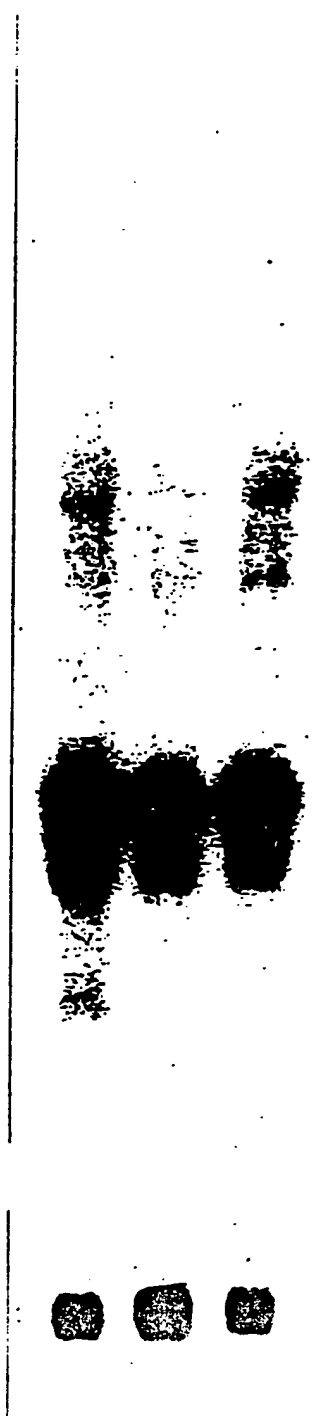
kb

-4.2

-3.5

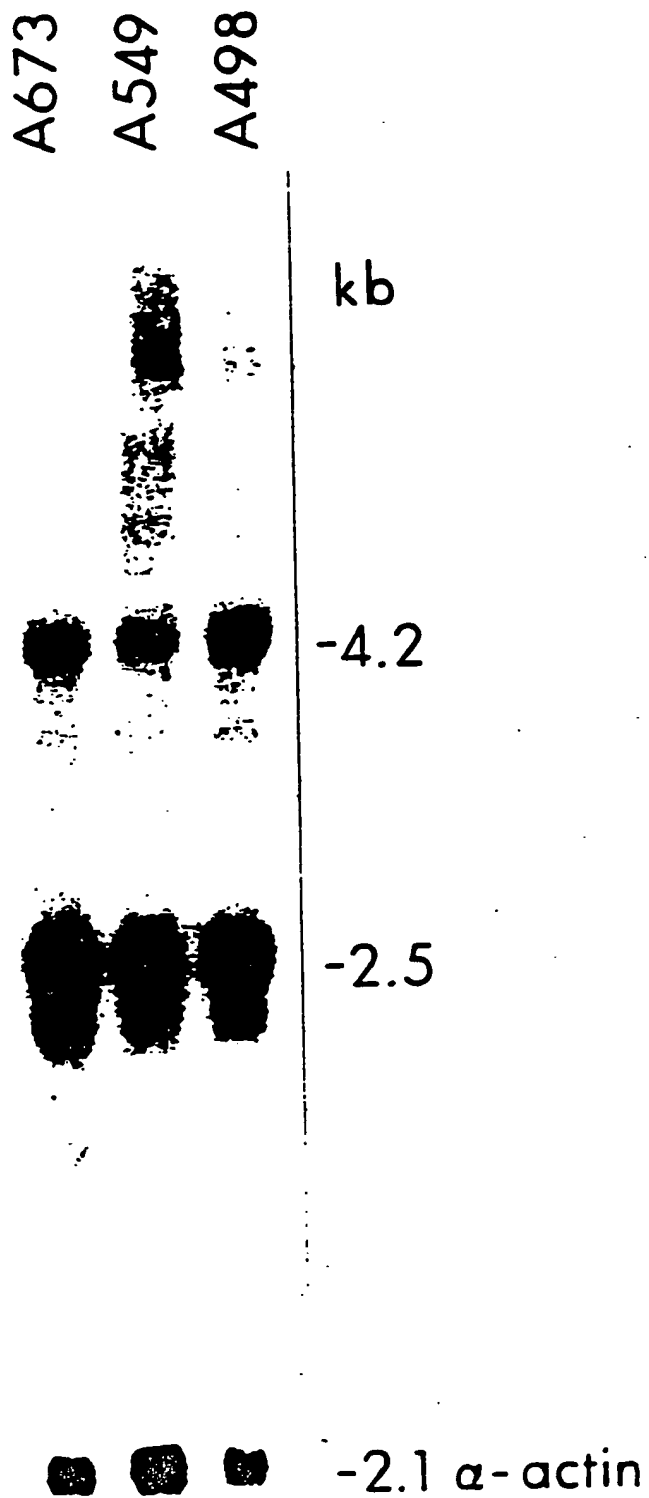
-2.5

-2.1 α -actin



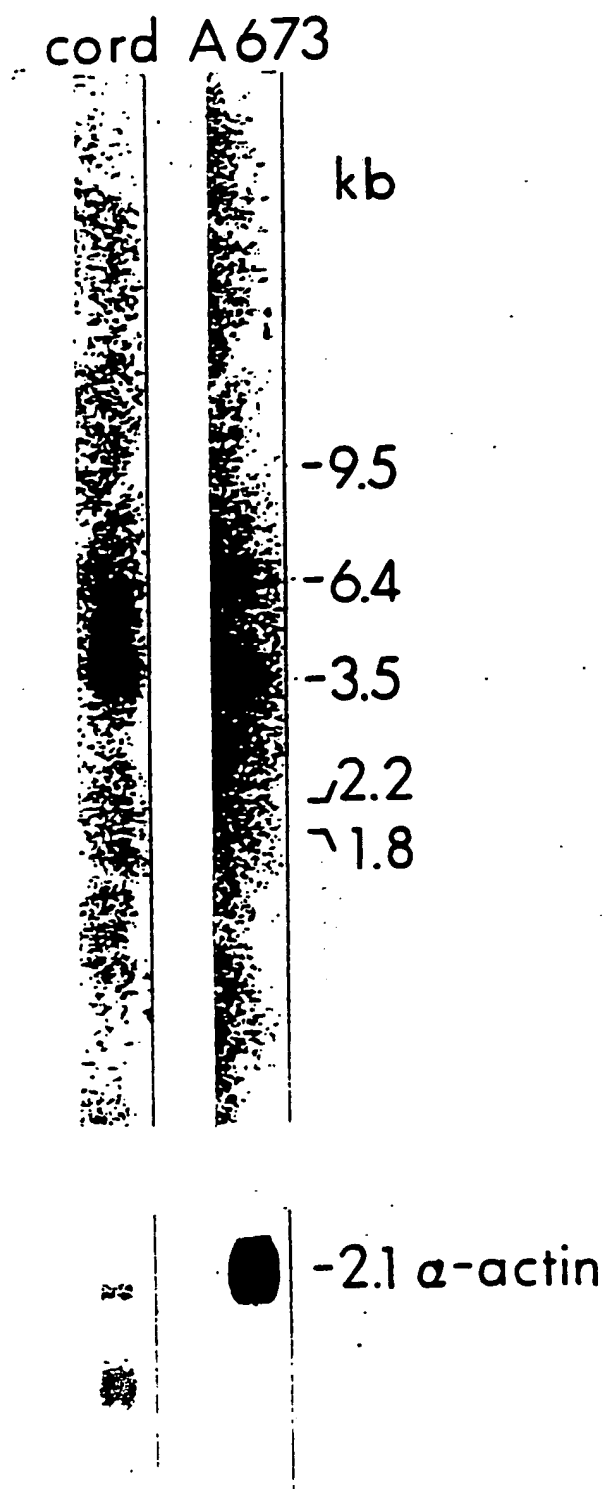
7/29

FIGURE 6



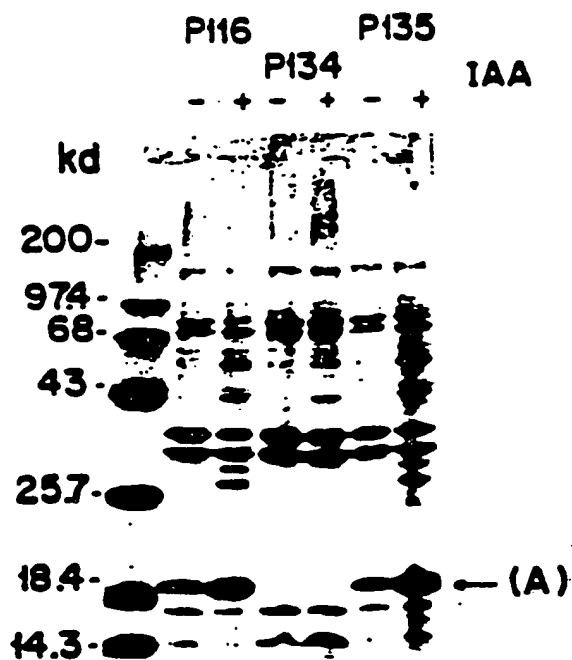
8/29

FIGURE 7



9/29

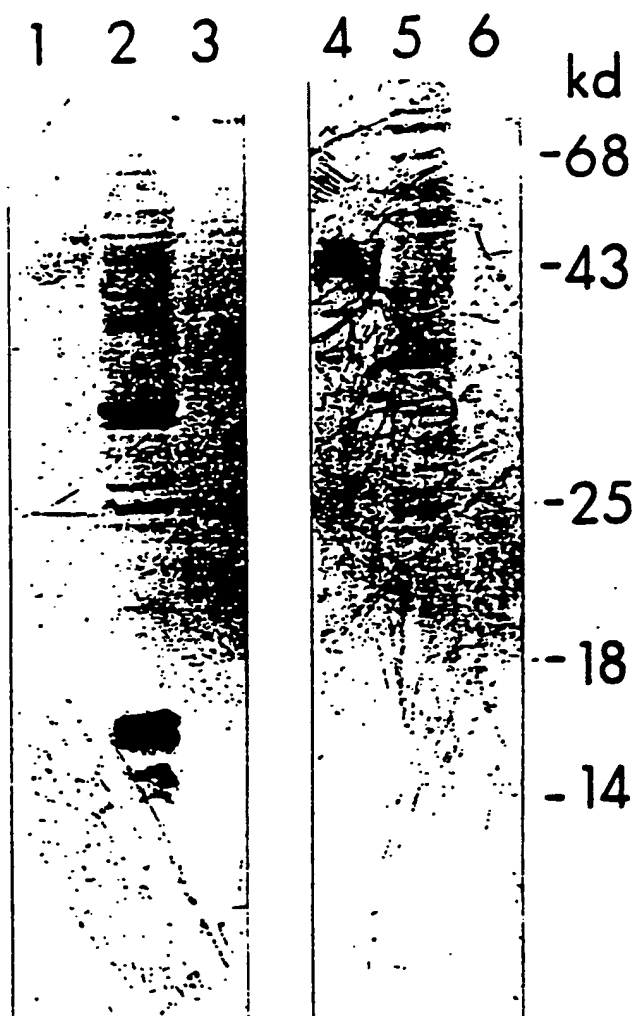
FIGURE 8



(A) = the protein having tumor growth inhibitory activity.

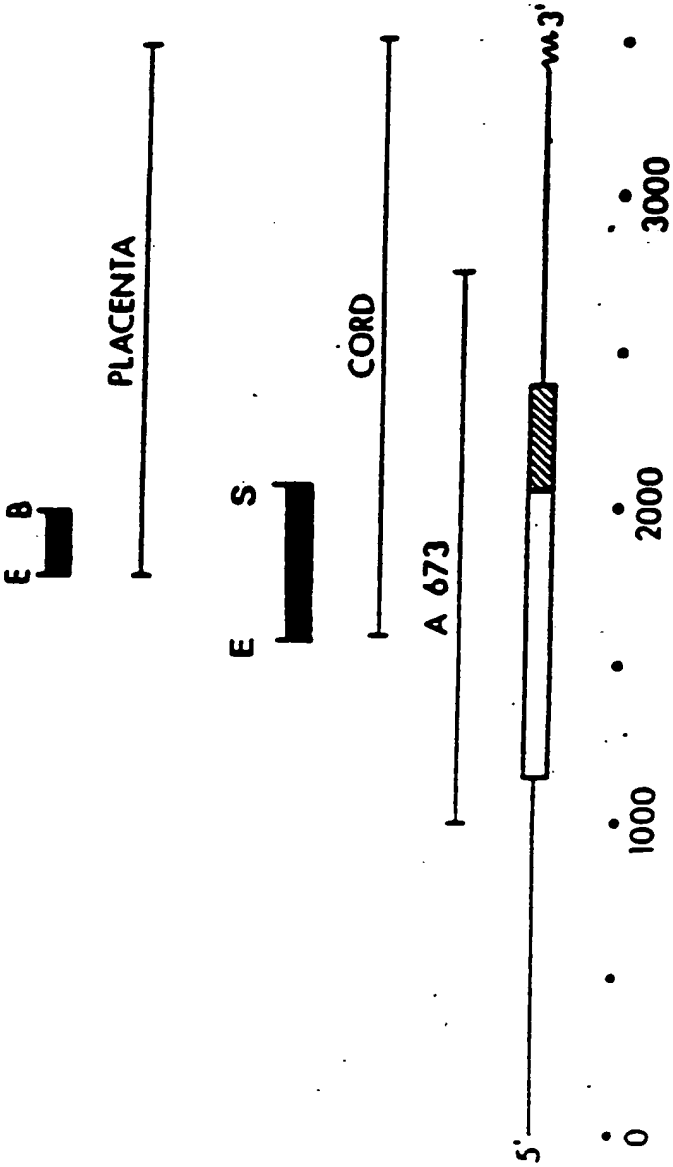
10/29

FIGURE 9



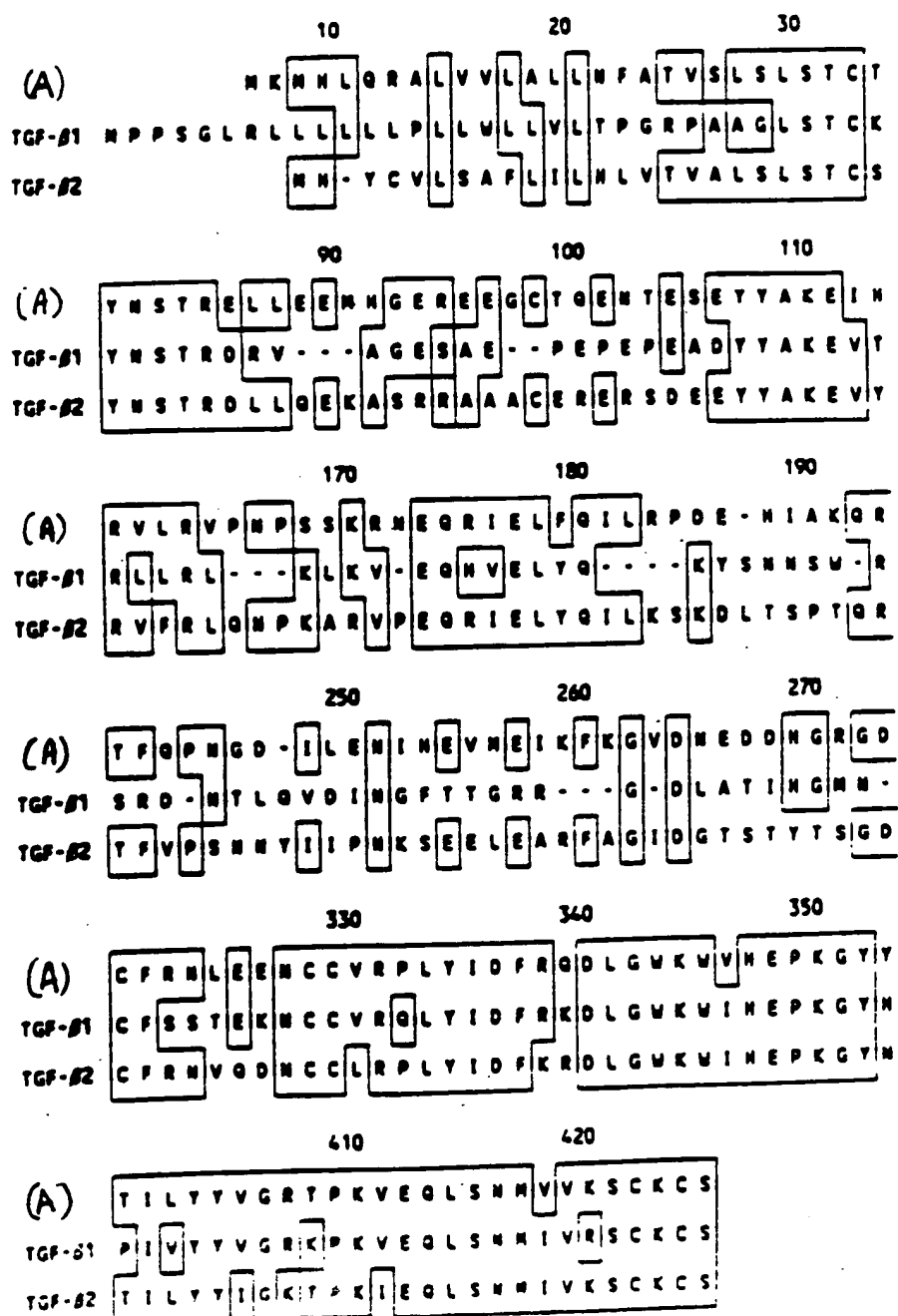
11/29

FIGURE 10



12/29

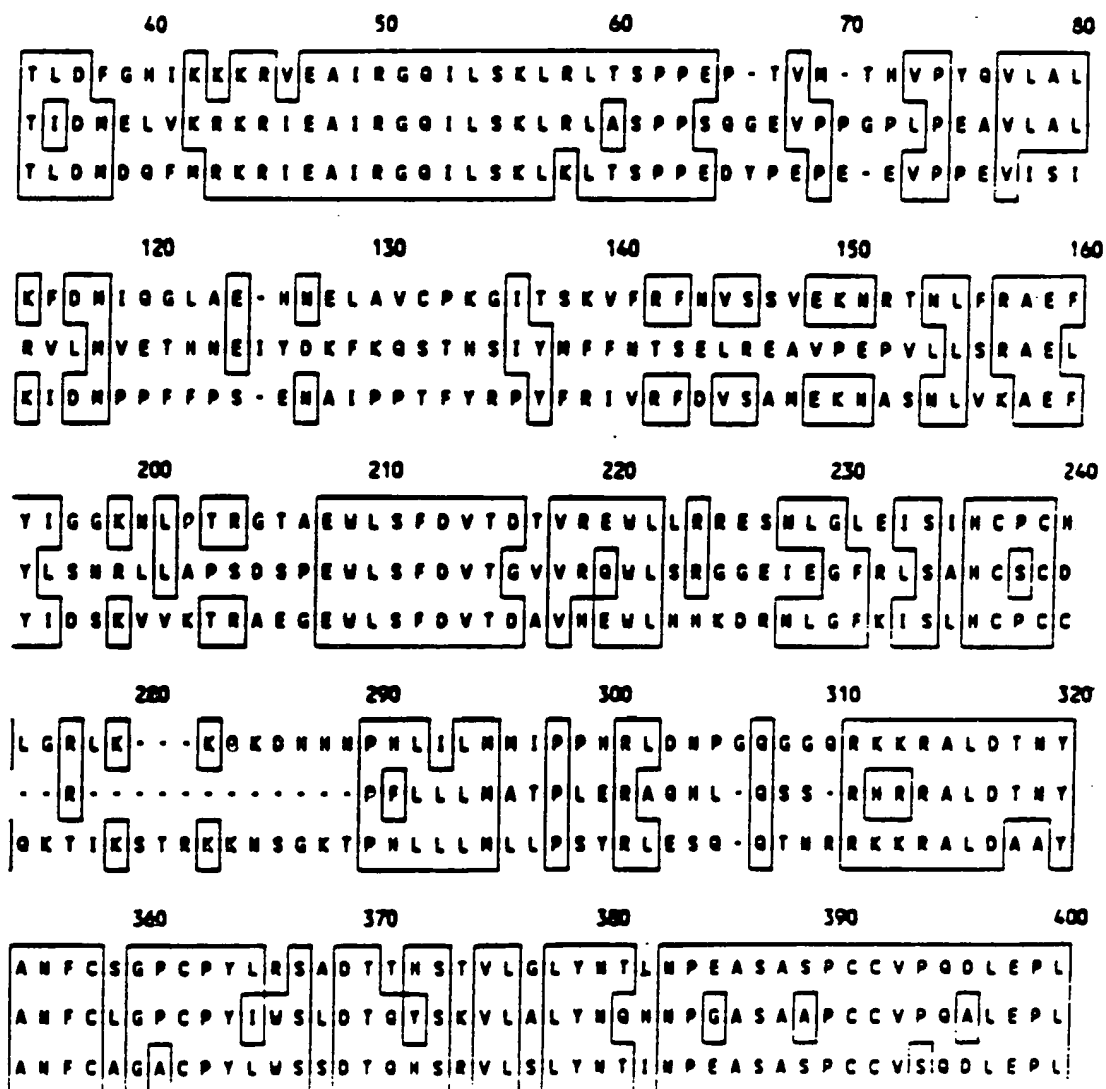
FIGURE 11



13/29

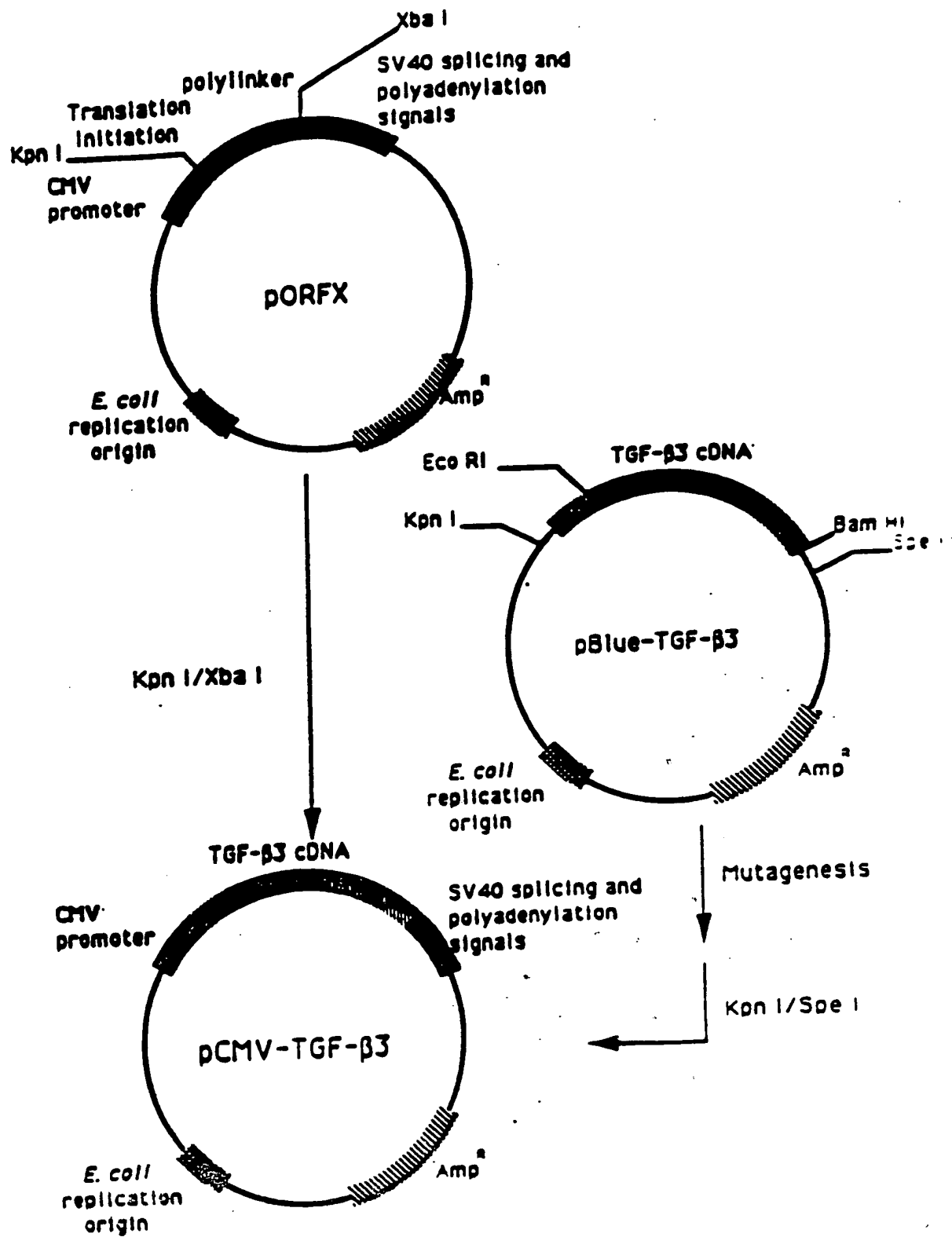
FIGURE 11

-continued-



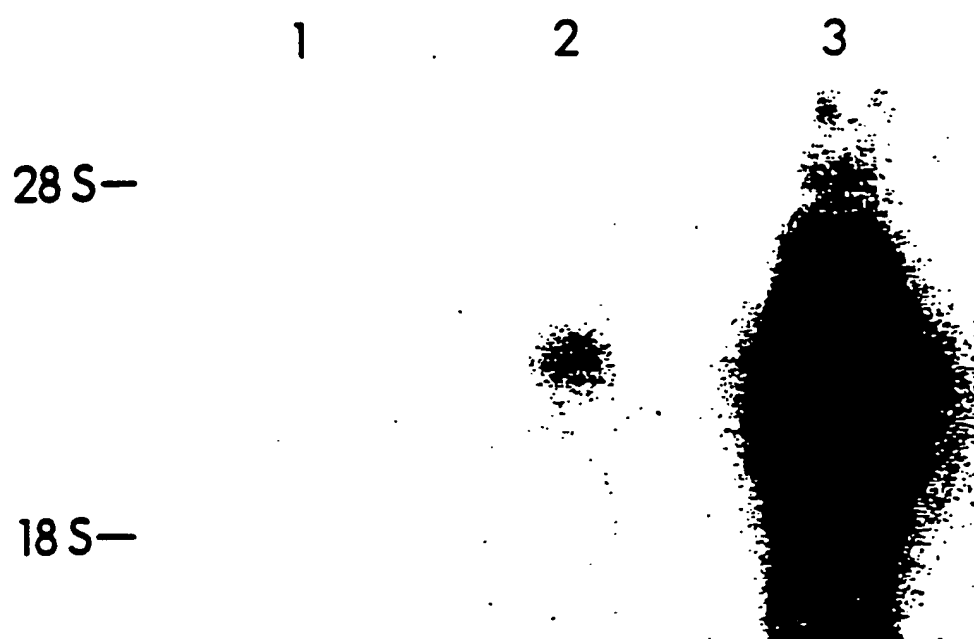
14/29

FIGURE 12



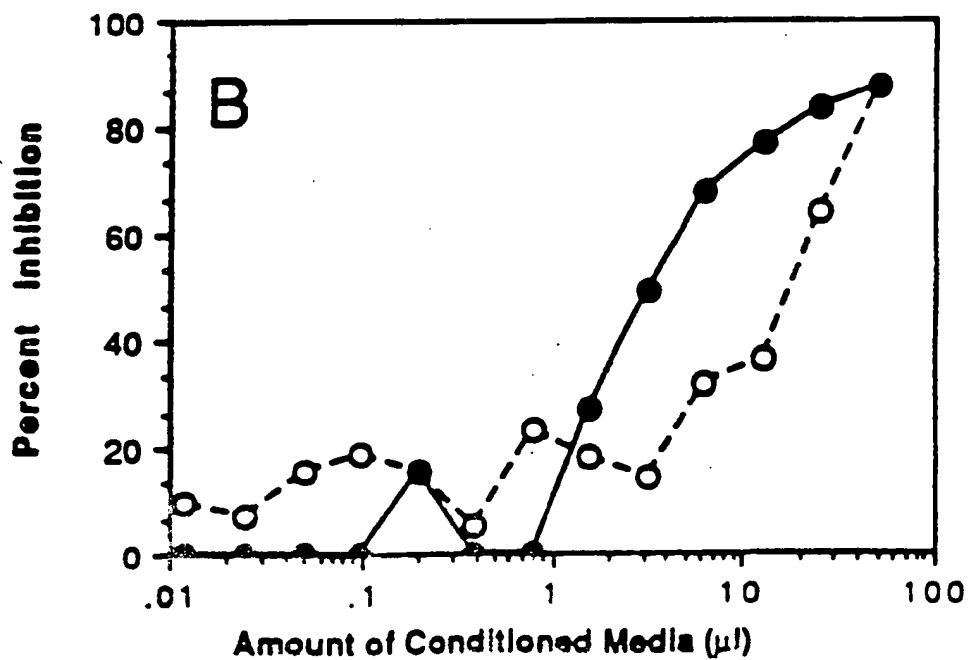
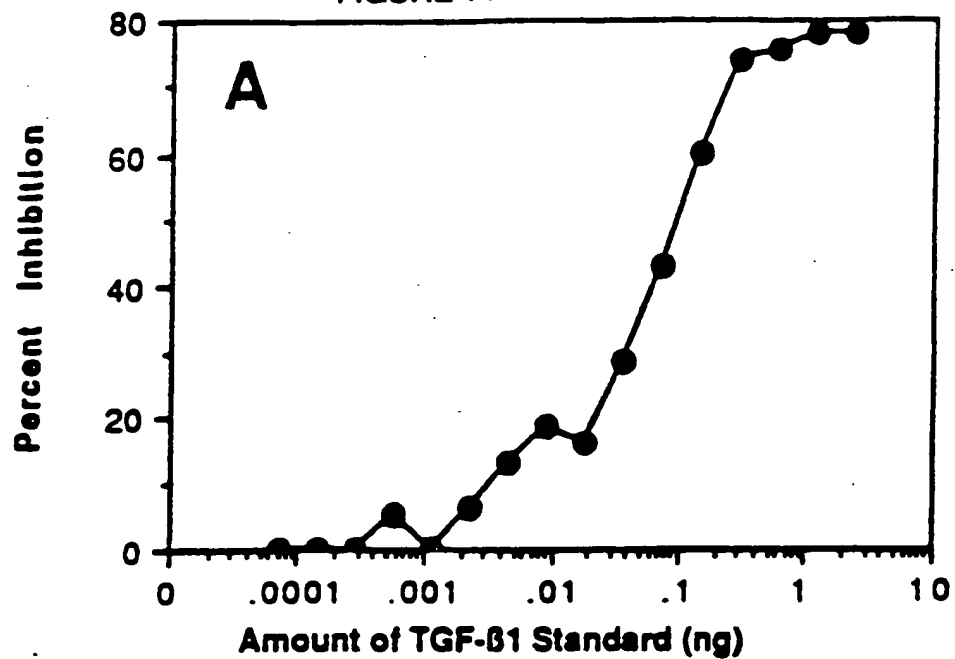
15/29

FIGURE 13



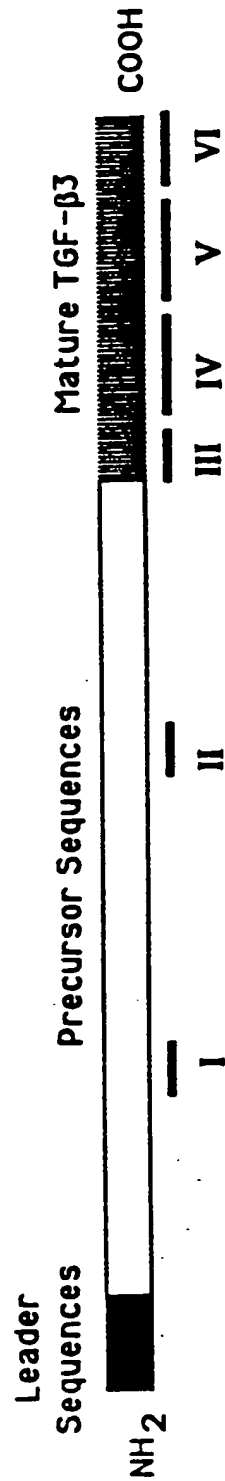
16/29

FIGURE 14



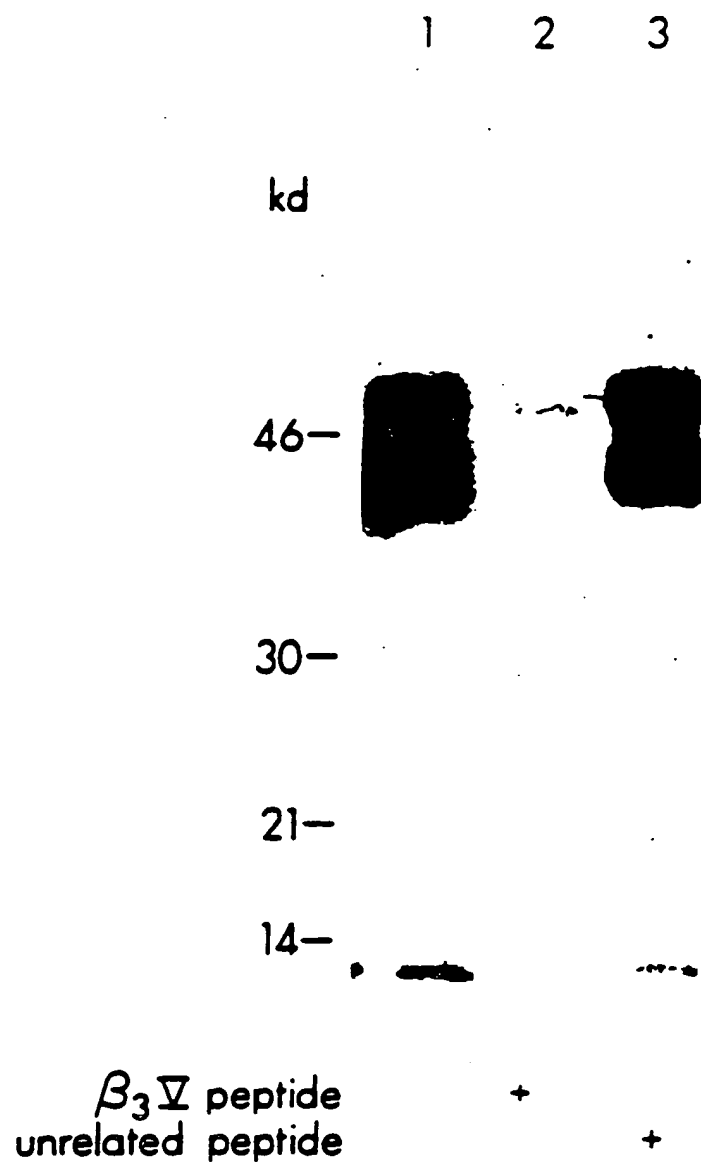
17/29

FIGURE 15

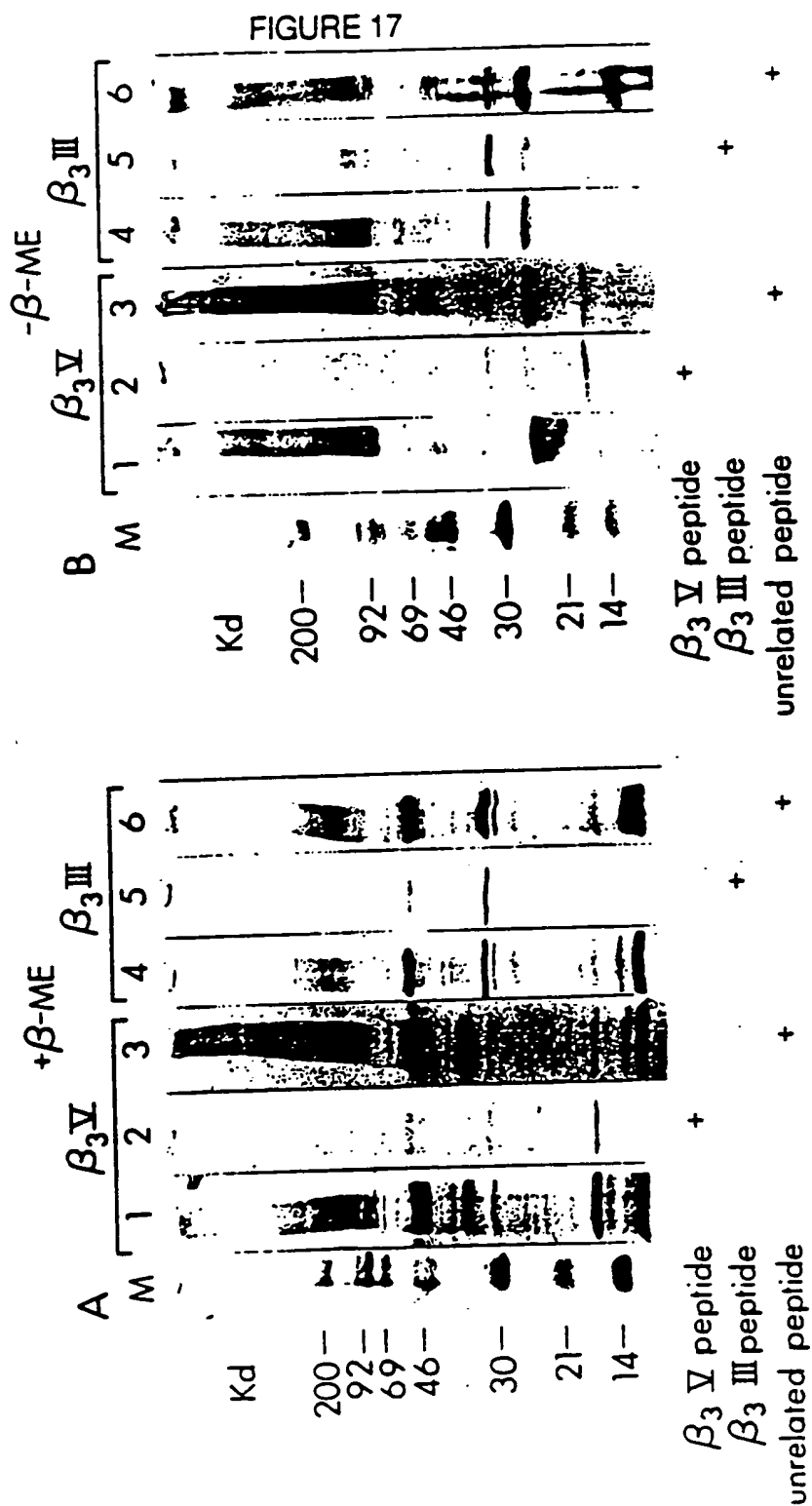


18/29

FIGURE 16

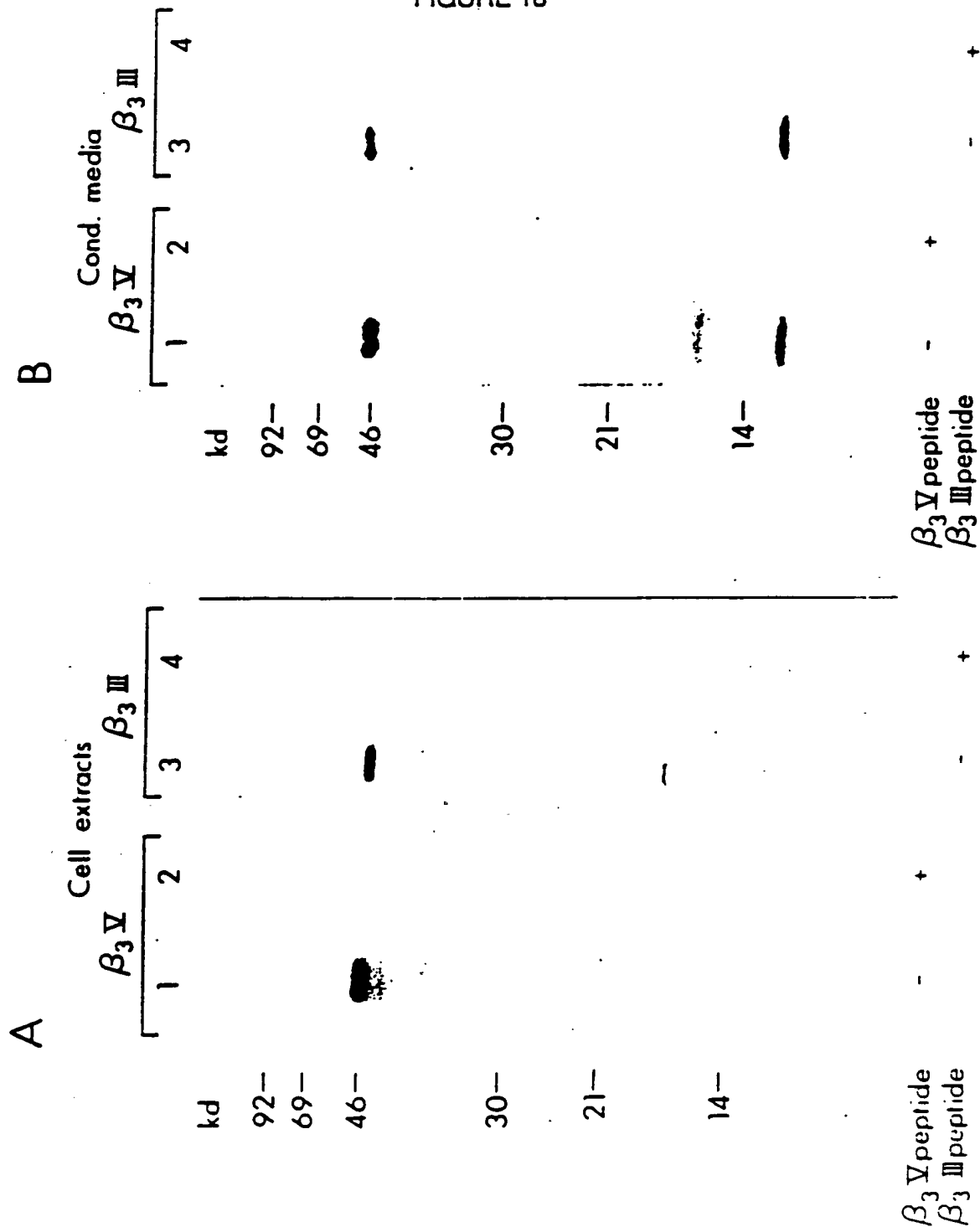


19/29



20/29

FIGURE 18



21/29

FIGURE 19A



22/29

FIGURE 19B



23/29

FIGURE 19C

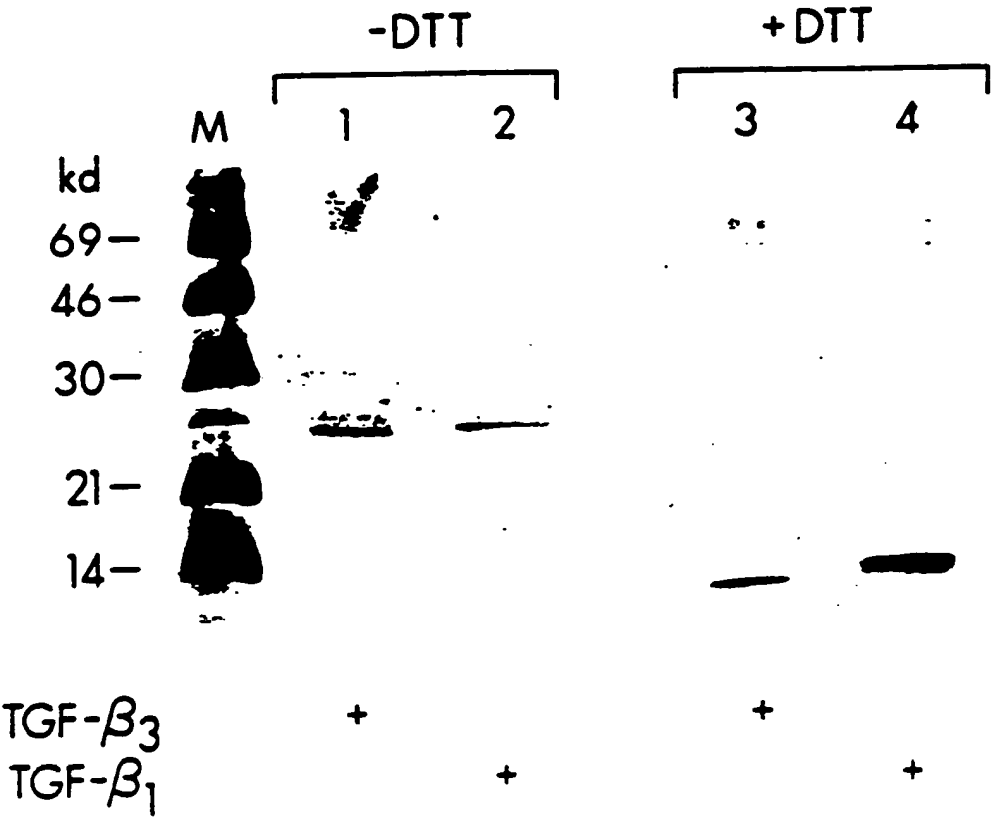


24/29

FIGURE 19D

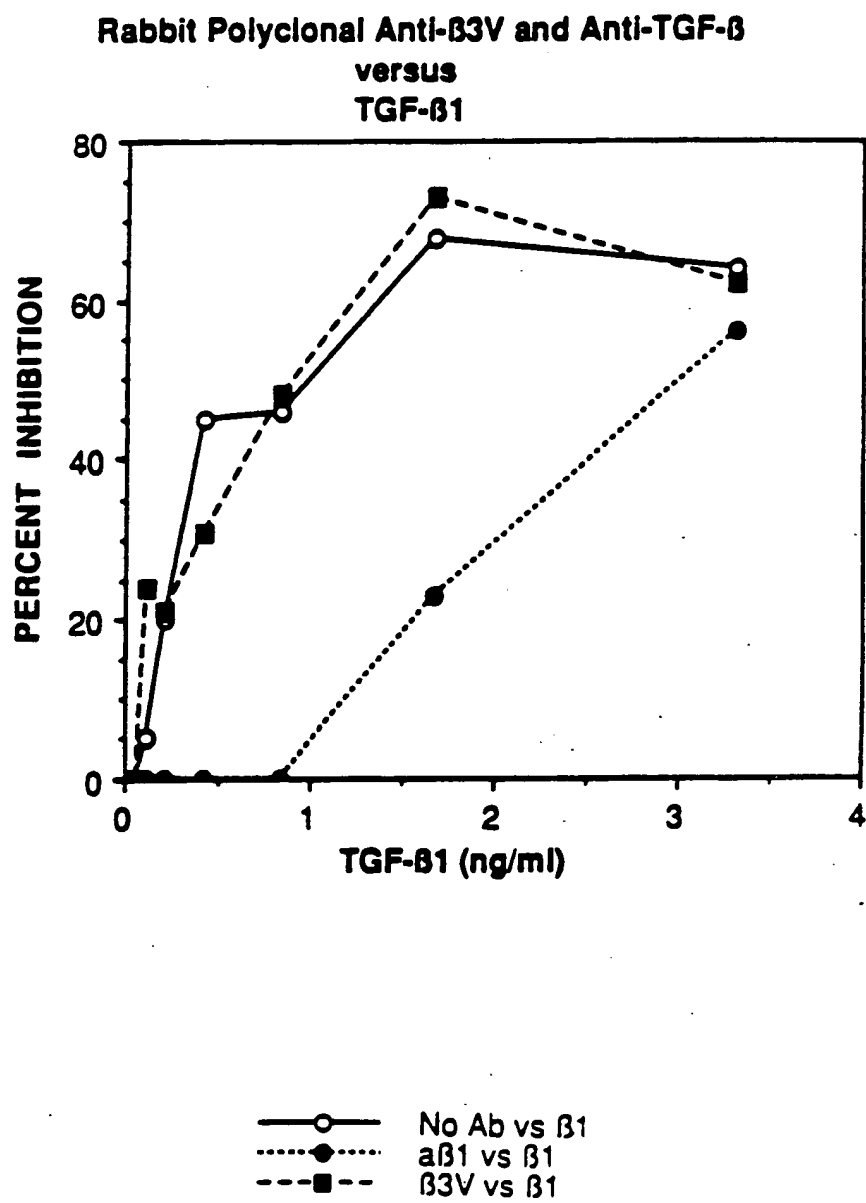


FIGURE 20



26/29

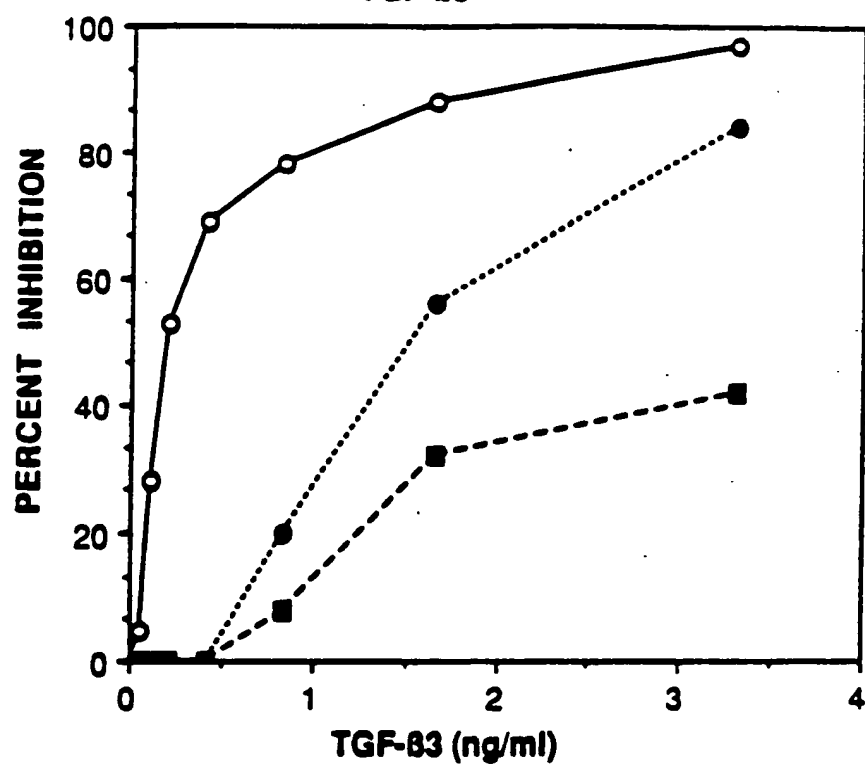
FIGURE 21A



27/29

FIGURE 21B

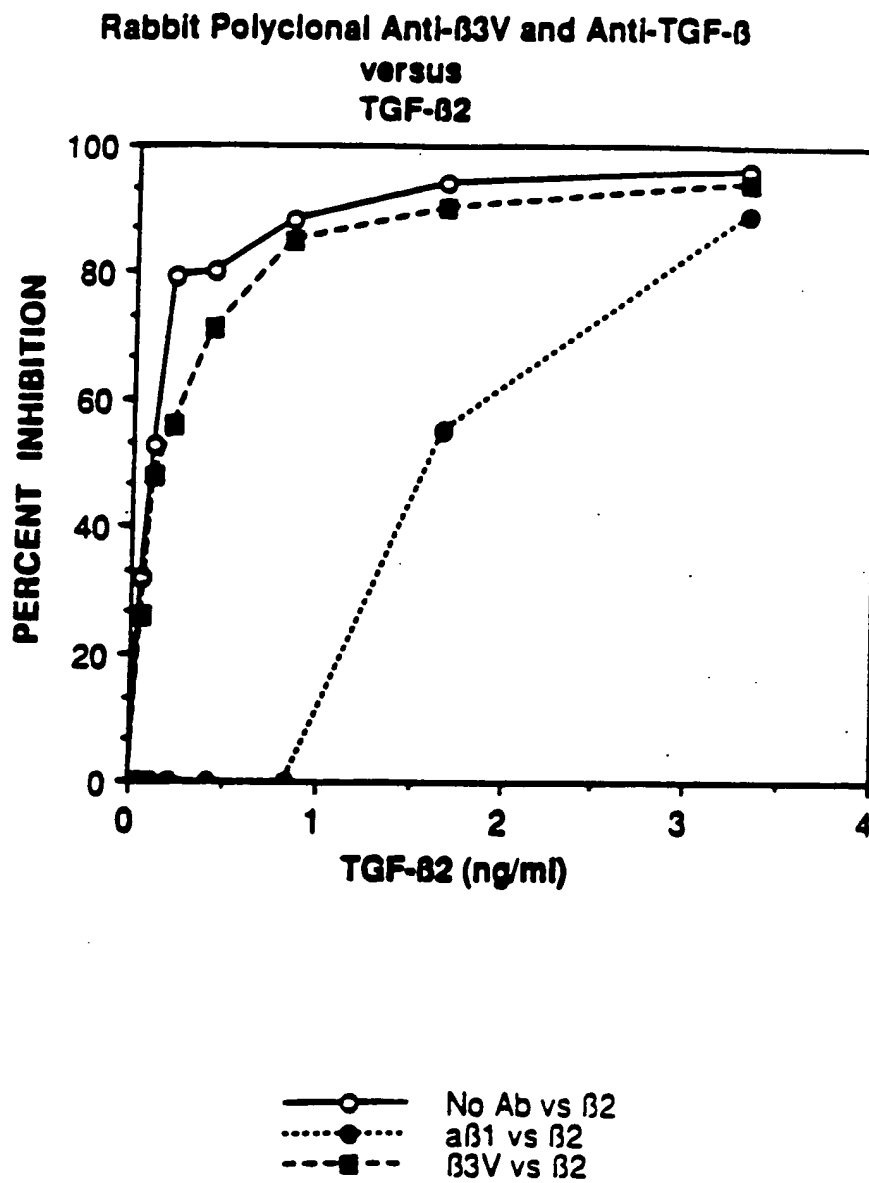
Rabbit Polyclonal Anti- $\beta 3$ V and Anti-TGF- β
versus
TGF- $\beta 3$



—○— No Ab vs $\beta 3$
.....●..... $\alpha\beta 1$ vs $\beta 3$
- - -■- - $\beta 3$ V vs $\beta 3$

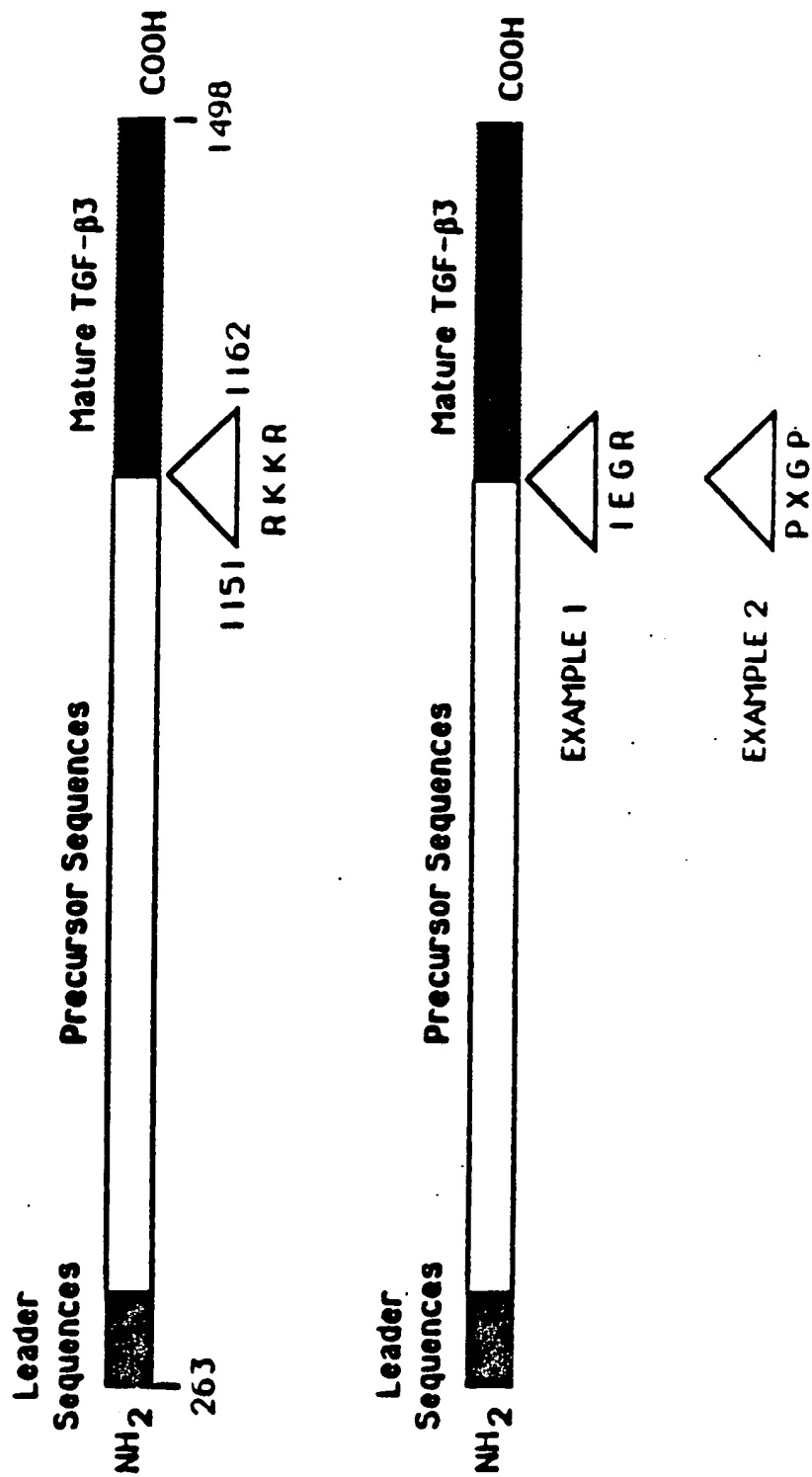
28/29

FIGURE 21C



29/29

FIGURE 22



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04541

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): C07K 3/20, 3/24; C12P 15/00		
US. CL.: 530/413, 420; 435/69.4		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	530/412, 413, 418, 419, 420; 435/68.1, 69.1, 69.4, 70.1, 172.1, 172.3	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Databases: Dialog (Files 5,73,155); USPTO Automated Patent System (File USPAT, 1971-1991).		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ^a	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US,A, 4,708,948 (IWATA et al.) 24 November 1987, See entire document.	1-37
Y	US,A, 4,886,747 (DERYNCK et al.) 12 DECEMBER 1989. See entire document, particular Fig. 4a-4c and Example 6, column 23.	1-37
Y	The EMBO JOURNAL, Vol. 7, No. 12, issued 1988, Derynck et al., "A New Type of Transforming Growth Factor-B, TGF-B3," pages 3737-3743, See entire document, particularly Fig. 1 Sequence.	1-37
Y	US,A, 4,931,548 (Lucas et al) 05 June 1990, see entire document.	1-37
<p>^a Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
22 October 1991		13 NOV 1991
International Searching Authority		Signature of Authorized Officer:
ISA/US		Robert D. Budens

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹², specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-4, drawn to methods of purifying TGF-B3 antibodies.
- II. Claims 5-21 and 34-37, drawn to methods of producing TGF-B3 precursors.
- III. Claims 22-23, 28-33 and 36-37 drawn to methods of producing mutant TGF-B3.
- IV. Claims 24-27, drawn to processes for producing TGF-B3 using precipitation

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Vol. 85, issued July 1988, Ten Dijke et al., "Identification of Another Member of the Transforming Growth Factor Type B Gene Family," pages 4715-4719, See entire document, especially Figures 2 and 3.	1-37
X, P Y	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, Vol. 593, issued 26 JUNE 1990, Ten Dijke et al., "Molecular Characterization of Transforming Growth Factor Type B3," pages 26-42, See entire document.	<u>1-4</u> 5-37
Y, P	MOLECULAR AND CELLULAR BIOLOGY, Vol. 10, NO. 9, issued SEPTEMBER 1990, Ten Dijke et al., "Recombinant Transforming Growth Factor Type Type B3: Biological Activities and Receptor-Binding Properties in Isolated Bone Cells," pages 4473-4479, see entire document.	1-37
A	CANCER research, Vol. 45, issued JUNE 1985, IWATA ET AL., "Isolation of Tumor Cell Growth Inhibiting Factors from a Human Rhabdomyo-sarcoma Cell Line, " pages 2689-2694.	1-37
A	CANCER RESEARCH, Vol. 45 issued JUNE 1985, Fryling et al., "Two Distinct Tumor Cell Growth inhibiting Factors form a Human Rhabdomyosarcoma Cell Line," pages 2695-2699.	1-37

